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PB-262 241

Feasibility of Applying Field Ionization Mass Spectrometry to Pesticide Research

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Prepared for

Health Effects Research Lab, Research Triangle Park, N C

Nov 76

021094

PB 262 241

EPA-600/1-76-037
November 1976

Environmental Health Effects Research Series

FEASIBILITY OF APPLYING FIELD IONIZATION MASS SPECTROMETRY TO PESTICIDE RESEARCH

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Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/1-76-037	3. RECIPIENT'S ACCESSION NO.
4. TITLE AND SUBTITLE FEASIBILITY OF APPLYING FIELD IONIZATION MASS SPECTROMETRY TO PESTICIDE RESEARCH	5. REPORT DATE November 1976 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) R.L. Dyer, H. d A. Heck, A.C. Scott and M. Anbar	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Stanford REsearch Institute Menlo Park, California 94025	10. PROGRAM ELEMENT NO. 1EA615 11. CONTRACT/GRANT NO. 68-02-1799
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711	13. TYPE OF REPORT AND PERIOD COVERED First Annual Report 14. SPONSORING AGENCY CODE EPA-ORD
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Pesticides Herbicides Chemical analysis Ionization Mass spectroscopy Biochemistry	Parathion Trifluralin Methyl parathion	07 C 06 A

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED 20. SECURITY CLASS (This page) UNCLASSIFIED	21. NO. OF PAGES 89 22. PRICE

EPA Form 2220-1 (9-73)

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EPA-600/1-76-037
November 1976

FEASIBILITY OF APPLYING FIELD IONIZATION MASS SPECTROSCOPY TO PESTICIDE RESEARCH

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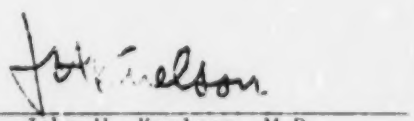
FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report presents data on the feasibility of measuring subnanogram quantities of pesticides in animal blood, tissues and excreta by use of nonradioactive isotope dilution analysis. Using multilabeled compounds as isotopic dilutants and nonfragmenting field ionization mass spectrometry (FIMs) as the analytical instrument, picomole (10^{-5}M) quantities of organic compounds can be quantitatively determined. This high sensitivity and quantitative accuracy permits the determination of pesticide residues in biological samples at very low levels. These data will provide a more substantiated assessment of persistence and degradation (biotransformation) of the compound and the potential hazard associated with exposure.



John H. Knelson, M.D.
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ABSTRACT

An isotope dilution methodology was developed for analysis of an insecticide, parathion, and a herbicide, trifluralin, isolated from rat tissues and excreta. Sample cleanup was facilitated by use of high-pressure gel permeation chromatography in conjunction with thin layer chromatography and reversed-phase high pressure liquid chromatography. Isotope ratio measurements were performed using multilabeled stable isotopic carriers and nonfragmenting field ionization mass spectrometry. Parathion and trifluralin were administered intraperitoneally and/or orally at the sub-mg/kg level, and the unchanged materials assayed in tissues and excreta at the ppb level. The technique was also applied to the determination of parathion and methyl parathion concentrations in aerosols. The biological implications of the results of the animal experiments are discussed.

CONTENTS

I INTRODUCTION 1

II EXPERIMENTAL 2

II A SYNTHESIS OF MULTILABELED CARRIERS 2

D_{10}-Parathion 2

D_7-Trifluralin 4

D_6-Methyl Parathion 5

II B EXTRACTION AND CLEANUP OF PTN AND TFN IN ANIMAL TISSUES 8

Extraction Methods 8

Cleanup Methods 9

Purification of PTN and TFN Following Cleanup 14

II C EXPERIMENTAL PROCEDURES 17

Animal Administration Regimens 17

Parathion IP 17

Trifluralin IP 17

Trifluralin Oral 18

Extraction Procedures 21

Gel Permeation Chromatography 23

High Pressure Liquid Chromatography 23

Thin Layer Chromatography 25

Mass Spectrometer Isotope Ratio Measurements 28

III RESULTS AND DISCUSSION 29

Cleanup of Extracts from Animal Tissues by High-Pressure Gel Permeation Chromatography 29

Analysis of Mass Spectrometric Data 30

Calculation of Isotope Ratio 30

Detection Limits 31
Background Correction and Error Analysis 35
Determination of PTN in Rat Tissues by Isotope Dilution 39
Determination of TFN in Rat Tissues by Isotope Dilution 44

IV DETERMINATION OF THE CONCENTRATION OF PTN AND MePTN IN AEROSOLS 53
Experimental 53
Results and Discussion 54

V CONCLUSIONS 57

REFERENCES 60

APPENDIX: RAW COUNT DATA 62

LIST OF FIGURES

1. FIMS Scan--D_{10}-Parathion 3
2. FIMS Scan--D_7-Trifluralin 6
3. FIMS Scan--D_6-Methyl Parathion 7
4. Separation of Benzene and Phenol by High-Pressure Gel Permeation Chromatography 13
5. Parathion Purification--Gel Permeation Chromatograms 24
6. Trifluralin Purification--Gel Permeation and Reversed-Phase High Pressure Liquid Chromatograms 26
7. FIMS Scans--Parathion: Comparison of HPLC and TLC Purification Techniques 32
8. FIMS Scans--Parathion 33
9. FIMS Scans--Trifluralin IP 34
10. FIMS Scans--Trifluralin Oral 36
11. Trifluralin Concentrations in Adipose Tissue and Liver of Rats Following IP Injection 51

LIST OF TABLES

1. Animal Data: Parathion IP Regimen 18
2. Animal Data: Trifluralin IP Regimen 20
3. Animal Data: Trifluralin Oral Regimen 22
4. Measured Parathion Levels in Rats 40
5. Summary--Parathion IP Results 41
6. Measured Trifluralin Levels in Rats--IP Administration 45
7. Summary--Trifluralin IP Results 46
8. Measured Trifluralin Levels in Rats--Oral Regimen 47
9. Summary--Trifluralin Oral Results 48
10. Inhalation Chamber Measurements 55

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Shirley Madan and Dr. Roger Barth of the SRI Life Sciences Division for their skillful assistance in performing the animal injections, feedings and necropsies. Mr. David Burkhouse was responsible for the synthesis of the multilabled materials. Mr. Russell Sperry and Mr. Gary Gonser carried out the mass spectrometric analyses. Mr. Albert Lobato and Mr. Ferdinand Engesser were responsible for construction of the gel permeation apparatus and for maintenance of the mass spectrometers. We especially thank Ms. Linda Morehead and Ms. Romen Rey for careful preparation of this manuscript.

I INTRODUCTION

This is the first annual report on a research program sponsored by the Environmental Protection Agency under Contract #68-02-1799.

The objective of this program has been to test the feasibility of determining very low quantities of insecticides and herbicides in animal tissues, urine, feces, and plasma by the use of nonradioactive isotope dilution analysis. Using multilabeled organic molecules as isotopic dilutants and nonfragmenting field ionization mass spectrometry (FIMS) as the analytical instrument, picomole quantities of organic compounds can be quantitatively determined. This very high sensitivity of detection may allow the determination of pesticide residues in animal tissues at much lower levels than has been heretofore possible. Such data will provide a more substantiated assessment of the long-term persistence and potential hazards involved in exposure of animals or humans to toxic chemicals.

During the first year of this research program, we have used the technique of isotope dilution analysis to study the distribution and persistence of parathion (PTN) and trifluralin (TFN) in rat tissues, plasma, urine, and feces following low dose injection of either compound or repeated oral administration in the case of TFN. The high sensitivity of the technique enabled us to determine ppb concentrations of PTN or of TFN in most of the biological materials examined.

Section II A describes the syntheses of multilabeled PTN, methyl PTN and TFN. Section II B contains a review of the new methodologies developed for sample cleanup by high-pressure gel permeation chromatography. Isotopic ratios were measured by multiscanning field ionization mass spectrometry using a cooled sample probe. The animal experiments and the details of the experimental procedures are summarized in Section II C. Results are compiled, discussed and compared with the literature in Section III. Section IV describes a brief experiment using isotope dilution techniques to measure the concentration of PTN and methyl-PTN in aerosols. Section V contains the conclusions of our study.

II EXPERIMENTAL

II A SYNTHESIS OF MULTILABELED CARRIERS

d_{10}-Parathion

The synthetic route was:

$$C_2D_5OH + SPCl_3 \xrightarrow{py} SP(OC_2D_5)_2Cl \quad (I)$$

$$I + p\text{-}NO_2phOH \xrightarrow{\Delta} NO_2\text{-}C_6H_4\text{-}O\text{-}P(=S)(OC_2D_5)\text{-}OC_2D_5 \quad (d_{10}\text{-parathion})$$

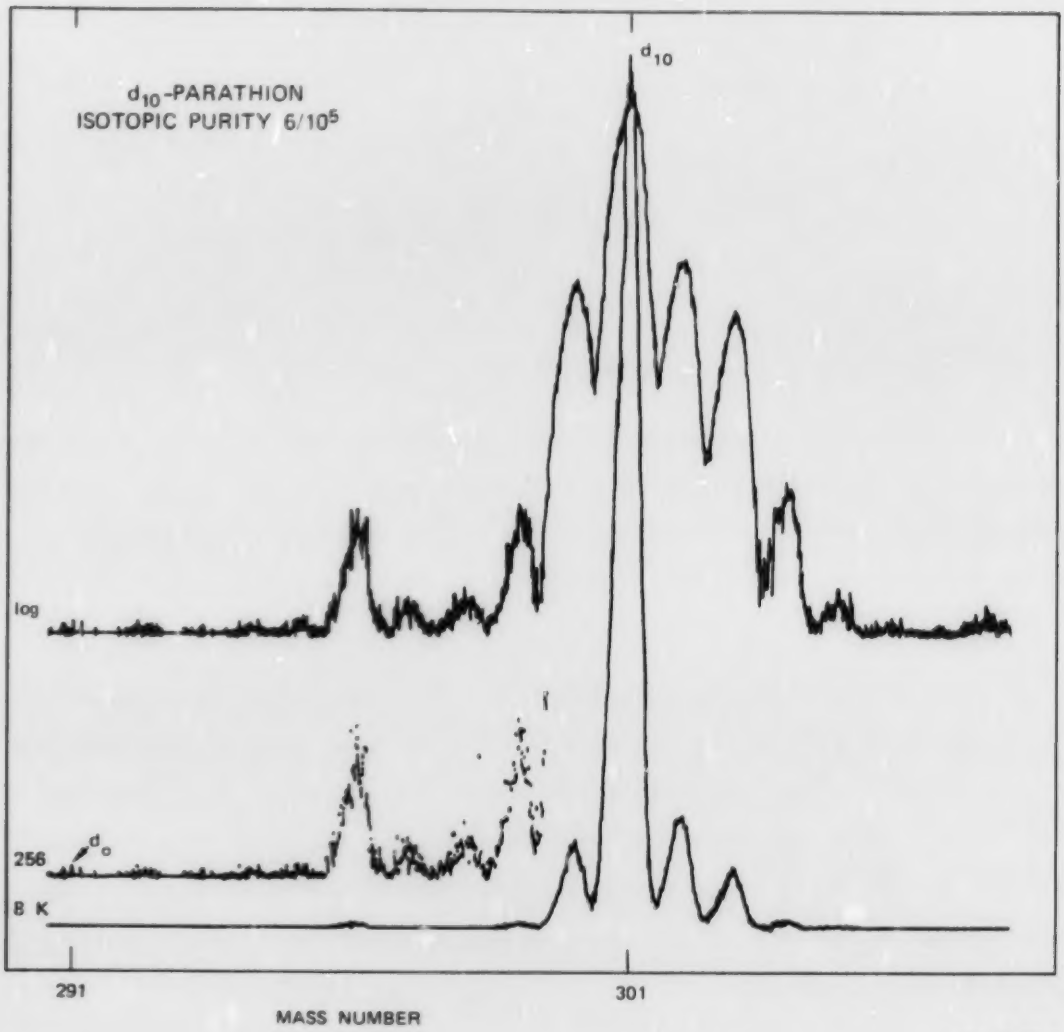
O,O-diethyl-d_{10}-chlorothiophosphate (I) was prepared by addition of 5 g d_5-ethanol in pyridine and benzene to 9.3 g thiophosphorylchloride in benzene at 0-20°C.[1] Pyridine hydrochloride was filtered and washed with benzene, the filtrate and washings condensed, and 3.2 g of the product collected by distillation at 80-92°C/20 torr.

Parathion was prepared by refluxing I with 2.32 g p-nitrophenol and 18 g sodium carbonate in acetone for 4 hours.[2] The filtrate was dissolved in benzene, washed with aqueous sodium carbonate, and dried over sodium sulfate. The product (3.3 g, 65% yield) was distilled and collected at 45-50°C/0.03 torr. Identity of the product was confirmed by IR and NMR.

A field ionization mass spectrum of the d_{10}-parathion is shown in Fig. 1. The isotopic purity, i.e., the ratio of counts at mass 291 (unlabeled) to the total of counts at masses 299-304 (labeled), is 5.7×10^{-5}.

FIGURE 1

FIMS SCAN: D_{10}-PARATHION



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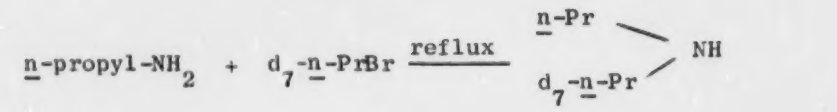
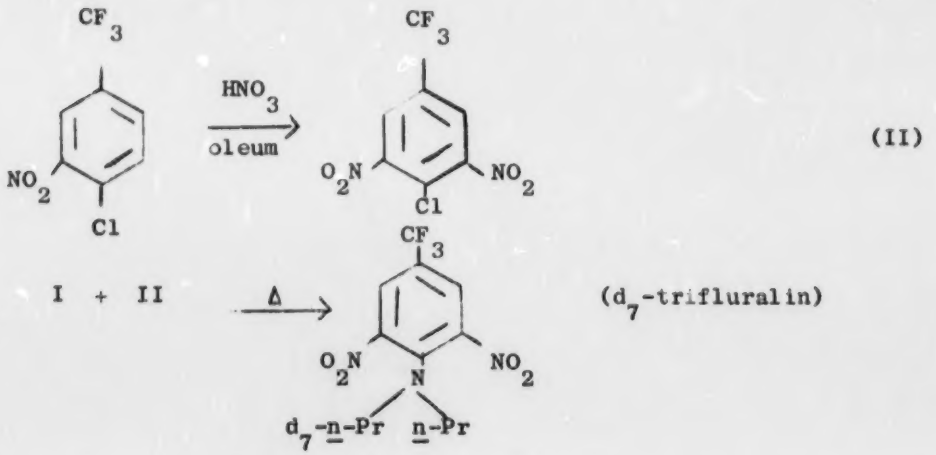
d_7-Trifluralin

The synthetic route was:



(I)



(II)

N-propyl-d_7-n-propylamine (I) was prepared by refluxing 4.4 g n-propyl amine with 5 g n-propyl-d_7-n-propyl bromide (Merck).[3] 4N sodium hydroxide was added and the amine distilled up to 110°C, yielding an azeotropic mixture. This was dried over NaOH pellets and distilled at 108-110°C, giving 3 ml of the desired product.

3,5-dinitro-4-chlorobenzotrifluoride (II) was prepared by dropwise addition of 80 g 96% H_2SO_4 and 60 g 90% HNO_3 to a mixture of 20 g 3-nitro-4-chlorobenzotrifluoride in 40 g 30% oleum.[4] The mixture was heated at 100°C for 3 hours and then 105-110°C for 30 min. The mixture was cooled, poured over ice, the precipitate filtered, washed and dried under vacuum. Recrystallization from ethanol gave an 85% yield of the product, melting point 58°C.

d_7-trifluralin was prepared by heating a mixture of 2.4 g II and 3 ml I at 100°C for 2 hours.[5] The mixture was dried with anhydrous ether, the amine hydrochloride removed by filtration and the filtrate concentrated to give 3 g crude product. Recrystallization from hexane gave 1.3 g (43%) of the final product. Identity was confirmed by melting point, IR and NMR.

A field ionization mass spectrum of the d_7-trifluralin is shown in Fig. 2. The material contains a significant amount of unlabeled contaminant at mass 335; the isotopic purity is only 1×10^{-3}.

The d_0 contaminant may be attributed to the presence of some unlabeled material in the d_7-n-propyl bromide (a reagent quality control problem we have encountered before). Because of the relatively high level of unlabeled material, which limits the dynamic range of the isotope dilution measurement, we administered the labeled material to the animals, using unlabeled trifluralin as carrier - the reverse of the parathion procedure.

d_6-Methyl Parathion

D_6-methyl parathion was prepared for use in the inhalation chamber experiments (Section IV). The synthetic route was:

$$CD_3OD + SPCl_3 \xrightarrow{NaOH(aq)} SP(OCD_3)_2Cl \qquad (I)$$

$$I + p\text{-}NO_2phOH \xrightarrow{\Delta} NO_2\text{-}C_6H_4\text{-}O\text{-}P(S)(OCD_3)\text{-}OCD_3$$

O,O-dimethyl-d_6-chlorothiophosphate (I) was prepared by dropwise addition of 10g d_4-methanol (Merck) to a cooled solution of thiophosphoryl-chloride, followed by 10 g of NaOH in 15 ml of water.[1] The reaction temperature was maintained from -20-0°C. The sodium chloride was dissolved in water and the organic layer separated. 7.0 g (14%) of the product was collected from a spinning band distillation at 58-60°/22 torr.

Methyl parathion was prepared by dropwise addition of 3.8 g of I to a mixture of 3.2 g p-nitrophenol, 2 g sodium carbonate, and 23 ml acetone, and refluxed for 4 hr.[2] The mixture was cooled, filtered, and the filtrate concentrated. 3.5 g (58%) of the product was recrystallized from methanol in dry ice-acetone. Identity was confirmed by IR, NMR and melting point.

A field ionization mass spectrum of the d_6-methyl parathion is shown in Figure 3. The isotopic purity is 2×10^{-5}.

FIGURE 2

FIMS SCAN: D_7-TRIFLURALIN

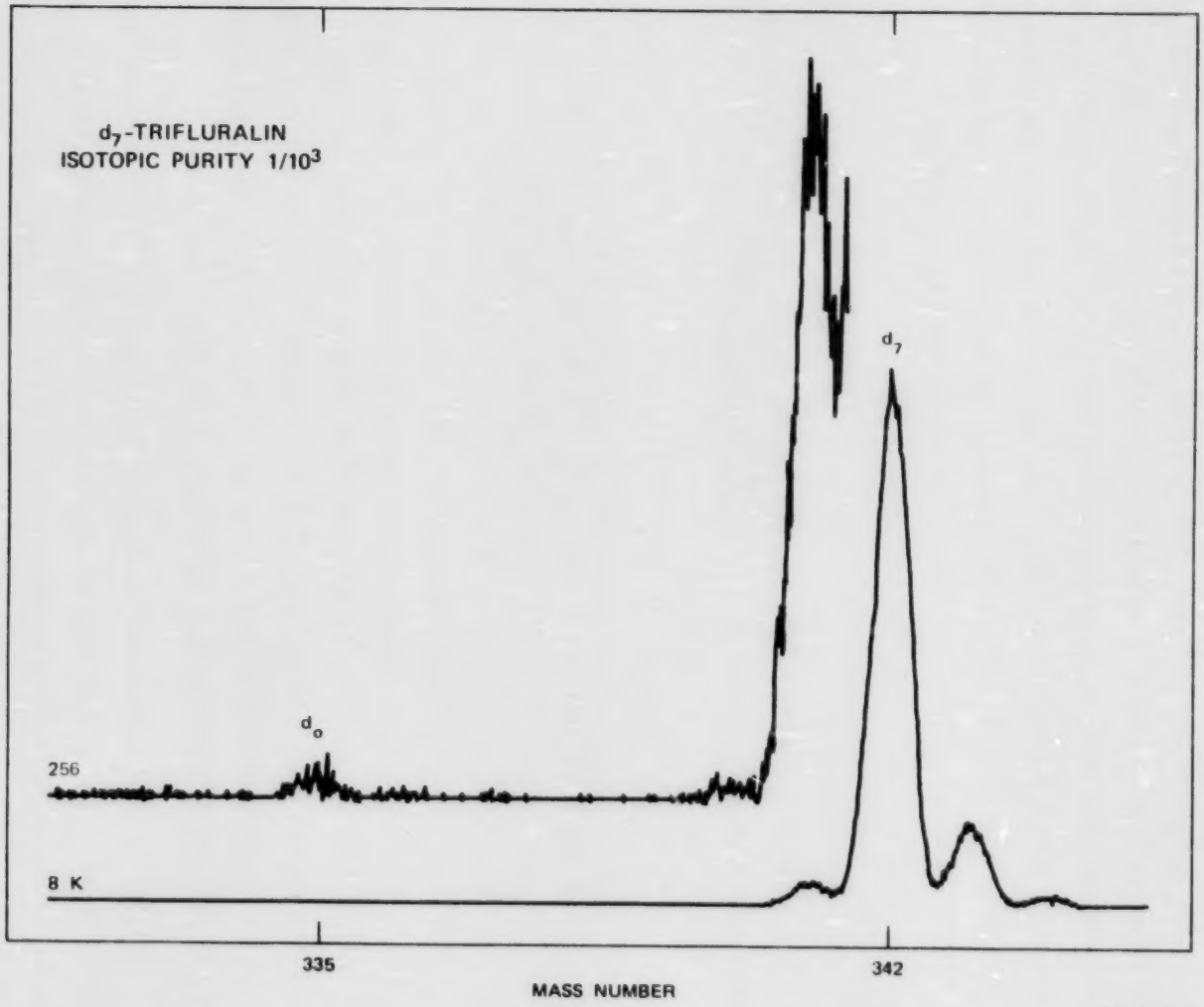


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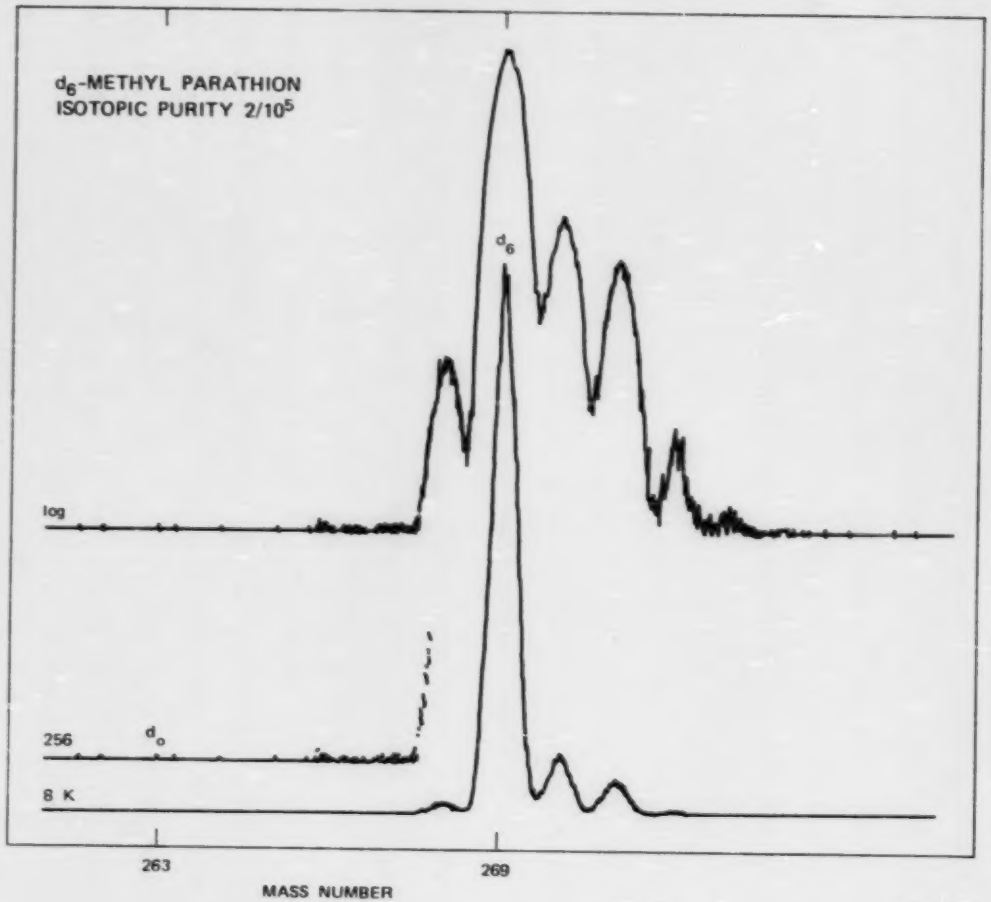
FIGURE 3

FIMS SCAN: D_6-METHYL PARATHION



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II B EXTRACTION AND CLEANUP OF PTN AND TFN IN ANIMAL TISSUES

The analysis of pesticide residue levels in animal and plant tissues generally includes the three phases of extraction, cleanup, and determination. The classical techniques of extraction and cleanup, summarized by Burchfield and Johnson[6] in 1965, continue to be widely used up to date. Major innovations, however, have arisen in the techniques for determination through the application of more sensitive or more rapid analytical tools, such as GC, HPLC, GC/MS, and isotope dilution/FIMS. The state of the art of many of these techniques as they apply to pesticide residue analysis, has been reviewed recently, e.g., J. Chromatog. Sci. 13, No. 5 and 6 (1975); Anal. Chem. 47, 157R (1975).

In the course of this study, we investigated a number of classical methods used for the extraction and cleanup of residues from lean and fatty animal tissues, bodily fluids, and feces. The goal in these investigations was to select or to develop efficient methods, broadly applicable to many tissues, that require a minimum of labor.

Although the conventional techniques were found to be effective, they are disappointingly slow and laborious, particularly the techniques used for cleanup of sample extracts. For this reason, a new approach was developed in which high-pressure gel permeation chromatography (GPC) was used as the first step in sample cleanup. Subsequent chromatography, either by TLC or reversed-phase HPLC, yielded samples having an exceptionally low level of chemical impurities. This section summarizes the various methods of extraction and sample cleanup that were tried during this research program.

Extraction Methods

Following Burchfield and Johnson,[6] samples of rat liver (typically 3-4 g) were initially ground in a mortar and pestle in the presence of about 3 g of Na_2SO_4. To test the efficiency of extraction, approximately

40 µg of TFN were added to several samples. In the classical methods, this step is usually followed by boiling the ground tissue in an organic solvent or by Soxhlet extraction. To avoid such time-consuming procedures, the ground tissues were homogenized in the presence of an organic solvent, either acetone or hexane (20 ml).

To reduce the time required for extraction, the initial grinding of tissue with Na_2SO_4 was omitted. Homogenization was carried out in the presence of 20 ml of hexane using a Tekmar Tissumizer. In addition to being less laborious, this method appeared to be more efficient, as the liver was more thoroughly dispersed in the organic solvent than was the case following Na_2SO_4 grinding. The Tissumizer technique was tested on a number of different tissues, as well as on feces, urine and plasma. Following cleanup (see below), analyses were performed by TLC. Moderately polar organic molecules, such as PTN and TFN, were extracted efficiently in all cases with 90% hexane/ether.

It is well known that homogenization of adipose tissue in the presence of organic solvents results in the extraction of substantial quantities of lipids. The same result is found, although to a much smaller extent, in the case of brain and liver. When such extracts are dried, an oily residue remains that cannot, in many cases, be effectively chromatographed, either by TLC or GC. Consequently, a cleanup procedure is required to remove the bulk of the lipids before chromatographic purification or analysis can be performed.

Cleanup Methods

The classical procedures of sample cleanup involve partitioning between polar and non-polar organic solvents, and/or sorption on various types of solid supports (e.g., charcoal, Florisil, siloxid) followed by batchwise or continuous elution.[6] A recent review suggests that no substantial alterations in these methods have been made within the past decade.[7] It seemed likely that the application of HPLC techniques, gel permeation chromatography in particular, in the area of sample cleanup might significantly reduce the time, labor and expense involved in this phase of the analytical procedure.

High-efficiency gel permeation columns appeared to offer many advantages as a first step in sample cleanup. Such columns achieve separation almost solely on the basis of molecular size. Since adsorption effects are minimal in such systems, the possibility that constituents in an organic extract would bind strongly to the column was deemed unlikely. It was expected, therefore, that gel permeation columns could be used for a large number of samples before column plugging or loss of resolution became severe.

During the first six months of this project, however, we did not have access to a gel permeation chromatograph. Although a system had been designed early in the project, various delays caused by the slow delivery of components and the construction of new chemistry laboratories postponed final acquisition of a working system until mid-winter. During this time, some of the classical techniques for sample cleanup were investigated, on the possibility that construction of the GPC system would require more time than expected.

The experiments that were carried out in this area involved partitioning trifluralin and parathion, after extraction from fat, liver, brain, or feces, between different organic solvents. The solvents used were hexane/acetonitrile, hexane/dimethylformamide, and hexane/methanol. As expected, the compounds of interest partitioned preferentially into the more polar phase (except for hexane/methanol), while the bulk of the lipid impurities remained in the less polar, hexane-rich phase. Although the partitioning ratios were favorable, it was found, in general, that at least two, and preferably three extractions using fresh polar solvent were required to thoroughly extract PTN and TFN from the hexane phase.

Concentration of the partitioned extracts to dryness revealed that, whereas most of the lipid materials had been removed by partitioning, the amount of oily residue remaining was still unacceptably high in the case of fat. If, however, the acetonitrile phase was first added to an aqueous

solution containing 5% Na_2SO_4, followed by re-extraction with hexane,[6] the extracts were found to be sufficiently pure to permit analysis by TLC. The same method was tried on several other tissues, and satisfactory results were obtained in all cases.

These procedures involving multiple extractions would have been extremely tedious had they been used for large numbers of samples. Cleanup by sorption on solid supports, which is normally used as an alternative or as an adjunct to cleanup by partitioning, would probably have been at least as time-consuming and was not, therefore, attempted. Work was directed instead toward the development of a successful GPC procedure.

A relatively inexpensive GPC system was constructed in this laboratory, the general design being based on that of Snyder and Kirkland.[8] Pumping is provided by a pneumatic amplifier pump (Haskel Engineering Co., Los Angeles, Model 28646-4) with a maximum pressure rating of 7000 psi. The pump is driven by filtered laboratory air, regulated at 80 psi. A second variable regulator at the pump reduces this pressure to approximately 22 psi, so that with a 46:1 pneumatic amplification, the output pressure is maintained at 1000 psi. This pressure provides a near-optimal flow rate in our gel permeation system.

For an isocratic gel permeation system, the Haskel pump provides many advantages. Its cost is low, and, in contrast to motor-driven, reciprocating pumps in the same price range, a stable rate of flow is maintained. This enables the Haskel pump to be used, if desired, with detectors that may be sensitive to fluctuations in flow rate or pressure, such as a refractive index detector. Moreover, the output of the Haskel pump is variable over a large range, so that it can be used for both analytical and preparative applications.

Prefiltered solvent at 1 atm pressure is gravity-fed to the pump through a 1/2" stainless steel tube. Outflowing solvent passes through 1/4" stainless steel tubing to a 35 micron line filter and then to a cross. Two arms of the cross are connected to high-pressure valves, one

of which carries solvent back to the solvent reservoir for rapid recycling and priming of the pump, while the other goes to waste. The remaining arm is connected to a 1/4"-1/16" reducer, thence through 1/16" tubing to a 15 micron line filter, a tee, and a third high-pressure valve. The third arm of the tee is connected to a flow-through, 0-5000 psi pressure gauge (American Chain and Cable, Helicoid Div., Bridgeport, Conn., Model 440L). The valves, filters, cross, and tee were purchased from High Pressure Equipment Co., Erie, Pa.

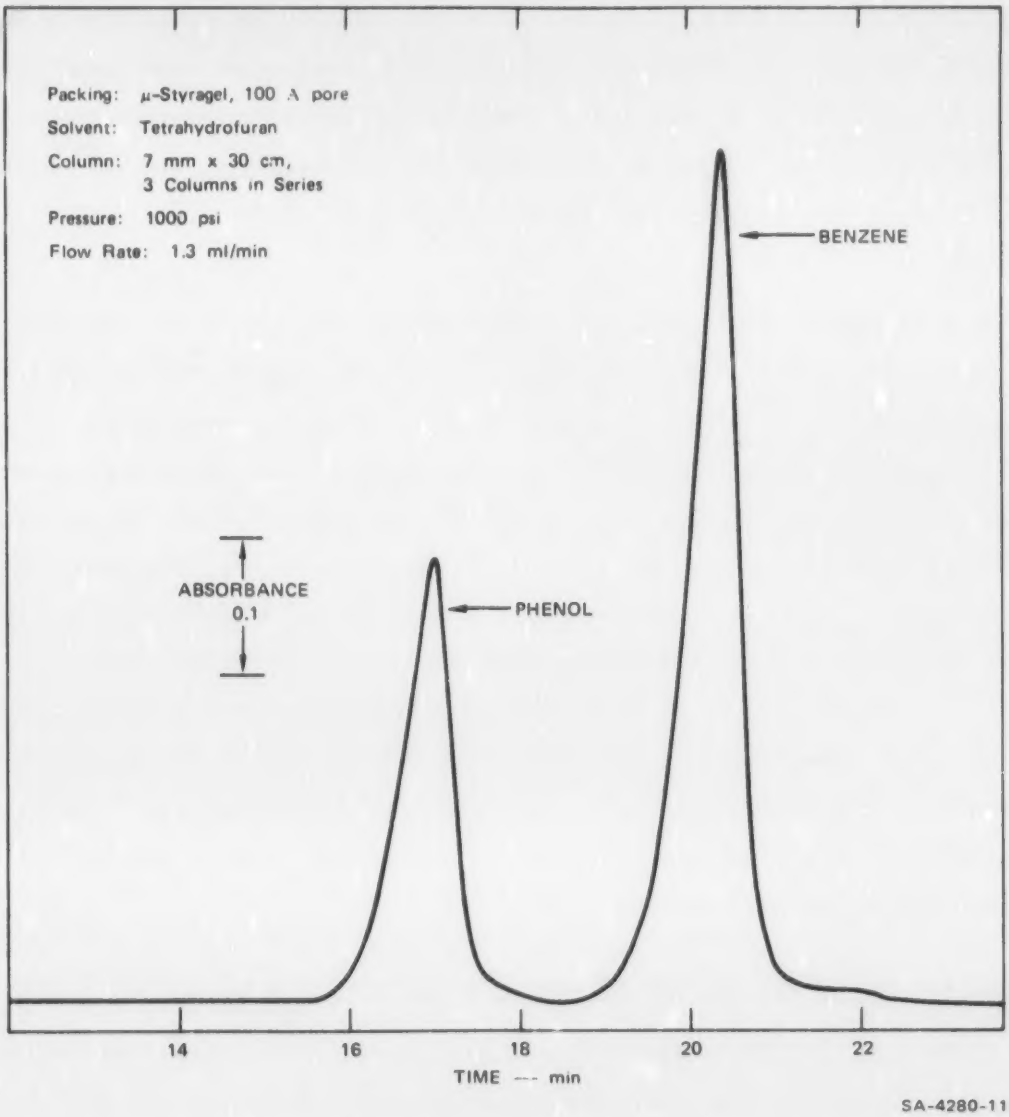
The sample injector (Rheodyne, Berkeley, Ca., Model 7105, with a 1.1 ml sample loop) follows the third high-pressure valve and is located as close as possible to the inlet of the first of three μ-Styragel columns, 100 Å pore (Waters Associates, Milford, Mass.), connected in series. At a pressure of 1000 psi, the flow rate through the system is approximately 1.25 ml/min, which is close to the optimum for gel permeation using 10 micron size particles.

A dual wavelength UV detector (Spectra-Physics, Berkeley, Ca., Model 230) connected to a dual channel strip chart recorder (Linear Instruments, Irvine, Ca., Model 285) monitors the absorbance of the column outflow simultaneously at 254 and 280 nm. Following the detector, a Rheodyne Teflon rotary valve (Model 50-11) is used either to manually collect an individual peak or to direct the flow of solvent to waste.

An example of the high resolution achievable with three μ-Styragel columns in series is shown in Fig. 4, which illustrates a size separation between benzene and phenol with tetrahydrofuran as solvent. Phenol, being the larger of the two molecules, is eluted first. The number of theoretical plates can be calculated from the equation $\underline{N} = 16\ (\underline{V}/\underline{w})^2$, where $\underline{V}$ is the elution volume and $\underline{w}$ is the baseline width of the peak in the same units. For this case, the calculated number of plates is 4800. The running time required for this separation was 20 minutes.

FIGURE 4

SEPARATION OF BENZENE AND PHENOL BY
HIGH-PRESSURE GEL PERMEATION CHROMATOGRAPHY



A very important feature of high pressure liquid chromatographic techniques for the cleanup of crude organic extracts is that large volumes of sample can be injected without significantly degrading the efficiency of the column. This property, unique to liquid chromatography, was pointed out by DeStefano and Beachell[9] for chromatography on silica and was reiterated by Moye.[10] We have investigated this point for the case of gel permeation. Acceptable bandwidths and resolutions were found with sample sizes as large as 1 ml, although 0.5 ml sample sizes gave somewhat better resolution. There was practically no difference in the bandwidth of peaks or in the resolution achieved in our GPC system for any size sample up to 0.5 ml.

To estimate the sensitivity of an isotope dilution assay following sample cleanup by GPC, a series of rat fat extracts were prepared containing various known amounts of unlabeled parathion and a known excess of the deuterated parathion as carrier. The extracts were chromatographed by GPC, the parathion peak collected, and the isotopic ratio determined by FIMS. Details of the chromatographic procedure are given below. The resulting mass spectra indicated that PTN could be determined in fat at the ppm level following a single gel permeation chromatography. Not surprisingly, however, gel permeation did not completely remove all of the potentially interfering impurities. It was evident that detection of PTN at lower levels in fat would require a further reduction in the amounts of these impurities. The final purification techniques used for PTN and TFN are described in the next section.

Purification of PTN and TFN Following Cleanup

Both TLC and HPLC were used as final purification techniques before mass spectrometric analysis. Purification by TLC on silica gel plates has been the standard method used for isotope dilution analysis in this laboratory.[11,12] Since an extensive literature exists on the TLC of PTN and TFN, the development of effective purification procedures was straightforward.

A Waters model 6000 high-pressure liquid chromatograph with gradient programming provided the capability to carry out efficient and rapid liquid chromatographic separations. High-pressure adsorption chromatography on silica is physically analogous to TLC, and the information learned from TLC is directly applicable. This chromatographic technique was not attempted, however, because of the tendency of silica to exhibit irreversible adsorption. Such an effect is expected with some components of a complex extract from animal tissues.

Preliminary experiments were performed in which the HPLC apparatus was used in a normal-phase partitioning mode for purification of PTN and TFN. In normal-phase liquid-liquid chromatography (LLC), the support is impregnated or bonded to a polar stationary phase, while a relatively non-polar solvent is used as the mobile phase. In reversed-phase LLC, the two phases are interchanged (i.e., less polar stationary phase, polar moving phase).

Normal-phase LLC appeared to offer one major advantage over reversed-phase LLC for the purification of volatile compounds. In normal-phase LLC, the compounds would be eluted in a non-polar, volatile solvent. Solvent evaporation would be the only step required before mass spectrometry. In contrast, in reversed-phase LLC, the compounds would be eluted in a non-volatile, mixed aqueous/organic solvent. Extraction into a volatile, organic solvent would be necessary before the samples could be concentrated to near-dryness.

Unfortunately, chromatography of PTN and TFN in a normal-phase mode was not successful. The solvent was cyclopentane/tetrahydrofuran in various proportions. Binding of the compounds to the stationary phase (μ-Bondapak CN) was too weak even in pure cyclopentane to enable this system to be used as a final purification procedure. This result was somewhat surprising, since partitioning of PTN and TFN between cyclopentane and the cyanopropyl stationary phase of μ-Bondapak CN was

expected to resemble partitioning of the compounds between hexane and acetonitrile (see above), and thus to favor retention of the compounds in a non-polar solvent such as cyclopentane. Despite its low polarity[13] and lack of miscibility with acetonitrile, cyclopentane is apparently a sufficiently strong solvent for PTN and TFN to overcome the expected solute-column interactions.

Reversed-phase LLC has been used successfully for the purification of pesticides, including PTN.[10,14] Mass spectrometric analysis of PTN following reversed-phase chromatography in 30% aqueous methanol solution indicated that the level of residual impurities, as measured by the background, was similar to that found following 2-dimensional TLC. The ratio of counts obtained at the unlabeled and labeled mass peaks of PTN following extraction of labeled PTN from tissues of control rats, was in the 10^{-4} range. Similar ratios have been obtained with other compounds following 2-dimensional TLC.[11,12]

The chromatographic techniques used do not remove all impurities. For instance, whether purification of PTN was by silica gel TLC or by reversed-phase HPLC, major impurities were found in the mass spectrum of PTN at m/e 297 and 298. These impurities do not interfere, however, with the determination of PTN, which has a molecular weight of 291. Since chromatography under the two sets of conditions is not expected to yield a common impurity, this impurity is probably solvent-related, since methanol was common to both purification techniques. It should be noted that the solvents used were of the highest grade commercially available (Burdick and Jackson Laboratories).

The background in TFN spectra, following reversed-phase HPLC in 30% aqueous methanol, was as much as four-fold lower for a given tissue than was the case with PTN. No detectable structure characterized the background in the TFN spectra obtained from diverse sources. With tissue of control rats, the ratio of counts at the masses of labeled and unlabeled TFN was in the 10^{-5} range. This indicates a very high level of purification achieved by the reversed-phase chromatography of TFN.

II C EXPERIMENTAL PROCEDURES

Animal Administration Regimens

Parathion IP

Rats were 50 day Sprague Dawley males (Simonsen Labs). Upon arrival, the rats were weighed, ear notched and housed in groups according to sacrifice schedule. The rats were allowed to acclimate for 3 days before treatment began.

2.6 mg of parathion (Pfalz & Bauer Chemicals) was taken up in 1 ml absolute alcohol and 25 ml sesame oil. This solution was injected by body weight, 100 µg/kg. Control consisted of 1 ml absolute alcohol (Gold Shield) and 25 ml sesame oil.

Treatment consisted of a single IP injection. There were 5 groups of 4 rats (1 control), sacrificed 1, 2, 4, 8 and 24 hr post-injection.

Fat, liver, brain, plasma, and red blood cells were collected from each rat at the time of sacrifice. The tissues were immediately placed in dry ice, the heparinized blood was immediately centrifuged, and the plasma and RBC cells separated and placed in dry ice.

Animal and sample weights are presented in Table 1.

Trifluralin IP

Rats were 60 day Sprague Dawley males (Simonsen Labs). The rats were allowed to acclimate for 4 days before starting the experiment. There were five groups of rats:

Group No.	No. of Rats	Sacrifice Schedule - Hours after Treatment
1	4	4
2	3	8
3	4	24
4	3	48
5	4	72

TABLE 1

ANIMAL DATA: PARATHION-IP REGIMEN

Group	Rat #	Body Weight		Dosage (ml)	Tissue Weight (g)			Fluid Vol. (ml)	
		1st day	necropsy		fat	liver	brain	rbc	plasma
1 hr sacrifice	1	260	260	0.26	3.0	10.5	1.7	1.5	1.8
	2	262	262	0.26	4.1	10.1	1.7	1.6	1.8
	3	266	266	0.27	4.0	10.3	1.7	1.7	1.5
	Control	264	264	0.26	2.2	12.2	1.8	1.2	1.5
2 hr sacrifice	1*	280	276	0.28	3.1	11.0	1.7	1.7	2.0
	2	282	280	0.28	4.2	12.8	2.0	1.8	2.0
	3	266	264	0.27	3.4	12.1	1.8	2.0	2.0
	Control	264	262	0.26	3.3	10.8	1.7	1.6	1.5
4 hr sacrifice	1	280	276	0.28	2.3	11.3	1.6	1.4	1.8
	2	262	254	0.26	2.9	10.2	1.9	1.1+	1.6+
	3	252	246	0.25	2.2	9.65	1.7	1.7	1.7
	Control	304	-	0.30	3.3	12.8	1.8	1.8	2.0
8 hr sacrifice	1	282	270	0.28	3.9	10.3	1.7	1.7	1.7
	2	282	268	0.28	3.2	11.2	1.8	1.7	1.8
	3	302	290	0.30	3.6	12.3	2.2	1.7	1.7
	Control	318	298	0.32	4.8	13.5	1.8	1.7	1.5
24 hr sacrifice	1	310	314	0.31	4.2	14.5	1.8	1.6	1.7
	2	320	320	0.32	3.4	14.1	1.4°	1.6	1.7
	3	296	290	0.30	6.3	11.4	1.9	1.7	1.7
	Control	296	296	0.30	3.9	14.3	1.6	1.8	1.8

* Parathion leakage at injection site

+ Centrifuge tube broke while centrifuging; blood poured into new tube and re-centrifuged. Plasma was pink from PBC lysing

° Not all of the brain was retrieved from the skull

Each rat received a single intraperitoneal injection of d_7-TFN, 500 µg/kg (500 µg/ml solution of d_7-TFN in sesame oil). Groups 1, 3 and 5 contained one control animal, injected with plain sesame oil, 1 ml/kg. Fat, liver and plasma were collected from each rat at the time of sacrifice. The tissues were immediately placed in dry ice and the heparinized blood was immediately centrifuged, the plasma taken off and placed in dry ice. In addition, urine and feces samples were collected from groups 3, 4 and 5 as below:

Gr. 3	0-24 hour samples
Gr. 4	24-48 hour samples
Gr. 5	48-72 hour samples

Animal and sample weights are presented in Table 2.

Trifluralin Oral

Rats were 60 day Sprague Dawley males (Simonsen Labs). The rats were allowed to acclimate for 4 days before the experiment began. The rats were housed 3 per cage in R3A cages and all animals received food and water ad lib.

The rats were divided into 4 groups of 3:

Group No.	Dosage
1 (Control)	Vehicle*
2	20 µg/kg
3	100 µg/kg
4	500 µg/kg

*9g NaCl, 4 ml polysorbate 80, 5 gm carboxymethylcellulose, 9 ml benzyl alcohol

Each rat received the compound daily orally for 21 consecutive days. An oral gavage needle (18 gauge from Popper and Sons, Inc. New York) was used attached to a 1 ml disposable syringe. The rats were weighed and the dosage adjusted to the correct amount with the d_7-TFN solutions made up at 20 µg/ml (Group 2), 100 µg/ml (Group 3) and 500 µg/ml (Group 4).

TABLE 2

ANIMAL DATA: TRIFLURALIN-IP REGIMEN

Group	Rat #	Body Weight		Dosage (ml)	Tissue/Fluid/Excreta Collected				
		1st day	necropsy		fat	liver	plasma (ml)	urine (ml)	feces (g)
4 hr sacrifice	1	332	322	0.32	2.9	14.7	1.7		
	2	332	322	0.32	3.3	14.3	1.6		
	3	356	346	0.36	5.0	14.4	1.8		
	Control	324	314	0.32	3.4	13.5	1.9		
8 hr sacrifice	1	374	378	0.37	6.1	19.0	1.7		
	2	384	378	0.38	5.2	18.3	1.7		
	3	348	342	0.35	4.6	13.4	1.7		
24 hr sacrifice	1	322	310	0.32	5.1	15.0	1.8	11.0	9.8
	2	318	328	0.32	3.2	14.3	1.5	8.5	10.0
	3	312	304	0.31	4.2	11.4	1.8*	7.5	9.3
	Control	348	350	0.35	5.6	18.7	1.8	11.5	11.0
48 hr sacrifice	1	342	332	0.34	4.9	15.5	1.8*	17.5	13.9
	2	318	308	0.32	5.4	13.7	1.6	12.5	11.4
	3	328	320	0.33	5.3	14.3	1.8	16.0	11.4
72 hr sacrifice	1	316	305	0.32	3.6	12.3	1.7*	13.3	17.1
	2	320	317	0.32	4.5	14.0	1.5	9.8	10.5
	3	336	340	0.34	7.5	16.2	1.6*	-+	10.1
	Control	334	336	0.33	3.6	16.4	2.0	14.5	11.8

* RBC lysing

+ 10 ml H_2O added to concentrated urine to collect sample

Urine and feces samples were taken from all groups on days 7, 14 and 21. The rats were placed individually in metabolism cages for 24 hours and returned to the R3A cage after the samples were collected.

The rats were sacrificed on the 22nd day after the treatment began. Fat, liver, plasma, urine and feces were collected at the time of sacrifice. Tissues were immediately placed in dry ice, the heparinized blood centrifuged, and the plasma removed and placed in dry ice. Animal and sample weights are presented in Table 3.

Extraction Procedures

Parathion: All tissues were kept on dry ice for 24 hours prior to extraction. One gram each of fat, liver, and brain, one ml of plasma and 1.1 to 2.0 ml of packed red blood cells were used per sample.

One ml of 1.5 M phosphate buffer was added to plasma and one ml of 1.5 M phosphate buffer plus 10 cc distilled water was added to the RBC samples to lyse the cells. All samples were homogenized in 8 ml of hexane:ether/90:10, using a Tekmar Tissumizer Model SDT, for 30 seconds at top speed, centrifuged (5 min, medium speed, clinical centrifuge), and the organic layer filtered through a millipore filter. Some plasma samples required ~0.5 cc iso-amyl alcohol to break a heavy emulsion. Twenty micrograms of d_{10}-PTN were then added as the labeled carrier to each sample. Samples were then dried down at room temperature under gentle nitrogen flow on a Meyer-N-Evap Organomation Analytical Evaporator and redissolved in 2.0 ml of tetrahydrofuran (THF) in preparation for the gel permeation chromatography.

Trifluralin: Fat, liver, and plasma samples were extracted using the same method as for parathion. Urine was treated the same as plasma. Feces were extracted as follows: One gram of feces was added to 8 ml distilled water and one ml of 1.5 M phosphate buffer in a 125 ml Erlenmeyer flask and allowed to stand for 30 min. to soften the feces.

TABLE 3

ANIMAL DATA: TRIFLURALIN-ORAL REGIMEN

Group	Rat #	Body Weight 1st day	Body Weight necropsy	Daily dosage (ml)	Tissue/Fluid Collected fat	liver	plasma (ml)	Urine Vol. (ml) 7 day	14 day	21 day	Feces Wt. (g) 7 day	14 day	21 day
Control	1	326	356	0.33	3.7	16.6	2.3	7.5	10.5	7.0	6.0	10.7	5.4
	2	330	332	0.33	4.6	15.1	2.2	12.5	13.5	20.0	5.2	9.3	8.3
	3	352	398	0.35	6.8	17.2	2.2	10.5	12.0	9.0	9.2	9.5	5.0
20 µg/kg	1	330	364	0.33	5.3	14.2	1.8	6.5	8.5	8.0*	8.4	8.5	6.4
	2	340	372	0.34	4.6	16.0	1.9	10.5	7.5	8.0	7.7	9.6	8.4
	3	350	378	0.35	9.3	15.4	2.0	10.5	10.0	11.6	8.0	5.0	5.7
100 µg/kg	1	362	366	0.36	8.1	15.5	1.4	12.5	12.0	15.0	11.2	10.0	8.7
	2	358	390	0.36	5.3	15.1	1.6	12.5	9.0*	9.5	7.7	8.4	7.5
	3	348	390	0.35	5.7	14.9	1.7	15.0	8.0	11.3	12.1	10.4	6.1
500 µg/kg	1	326	343	0.33	6.1	13.3	1.7	12.0	13.5	10.5	9.1	12.6	4.9
	2	344	364	0.34	5.3	15.6	1.8	7.0	6.5	10.0	9.5	9.5	8.4
	3	323	324	0.32	4.2	13.5	-+	11.0	15.0	9.0	5.0	5.7	5.2

* Urine contaminated with feces

+ Centrifuge tube broke; blood sample recentrifuged. Plasma was pink from RBC lysing

Eight ml of hexane:ether/90:10 were added and the mixture thoroughly shaken by hand for about 30 seconds. This mixture was then placed in a centrifuge tube and centrifuged for 5 minutes at top speed in a clinical centrifuge. The organic layer was removed and filtered.

All samples received 80 micrograms of unlabeled trifluralin as carrier, added just prior to homogenization-extraction, rather than after filtration. All samples were dried down at 30°C under a gentle N_2 stream and redissolved in 2.0 ml of THF prior to GPC.

Gel Permeation Chromatography

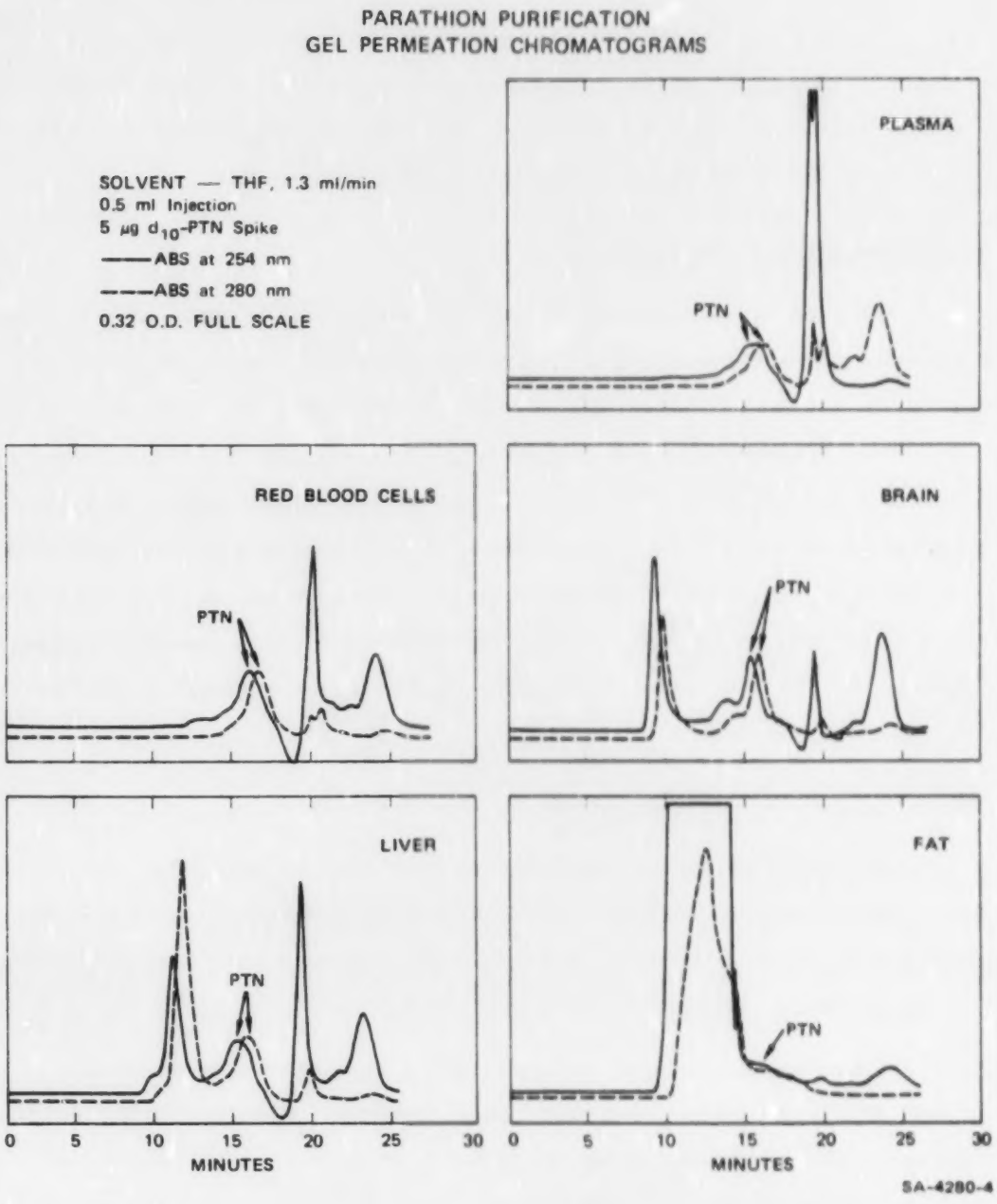
All samples were stored in Teflon-capped culture tubes at 5°C until gel permeation chromatography. Exactly 0.5 ml of sample was injected into the liquid chromatograph utilizing three 100 Å microstyragel columns in series. Conditions for operation were as follows: solvent - THF, pressure - 1000 psi., flow rate - 1.25 ml/min, chart speed - 0.5 inch/min. Parathion or trifluralin peaks were collected into another culture tube manually using U.V. absorbance readings. Final volume was approximately 2.5 ml of THF. Figure 5 displays representative gel permeation chromatograms for PTN. No attempt was made to identify the impurity components of the chromatograms.

Reversed-Phase High Pressure Liquid Chromatography

Preliminary parathion samples were processed by both HPLC and thin layer chromatography following GPC. Since TLC appeared to give cleaner mass spectra (see discussion, page 31), TLC was used for final purification of the parathion samples.

All trifluralin samples were processed in a Waters Assoc. Model M6000A HPLC with a Model 440 absorbance detector. The previously collected GPC sample was dried under a gentle N_2 stream as before and redissolved in 0.5 ml H_2O-MeOH/30:70.

FIGURE 5


PARATHION PURIFICATION
GEL PERMEATION CHROMATOGRAMS
SOLVENT — THF, 1.3 ml/min
0.5 ml Injection
5 µg d_{10}-PTN Spike
——ABS at 254 nm
– – –ABS at 280 nm
0.32 O.D. FULL SCALE
PLASMA
PTN
RED BLOOD CELLS
PTN
BRAIN
PTN
LIVER
PTN
FAT
PTN
0 5 10 15 20 25 30
MINUTES
0 5 10 15 20 25 30
MINUTES
SA-4280-4

Some fat samples would not dissolve in this solvent and were placed in 100% MeOH, shaken until all the TFN was dissolved, centrifuged to remove the fat globules, dried again under N_2, and redissolved in H_2O-MeOH. Conditions for operation for the HPLC were as follows: Solvent 30% H_2O - 70% MeOH, flow rate - 2 ml/min., pressure - ~ 5000 psi., column - micro - Bondapak C_{18} reversed phase, chart speed 0.5 inch/min. absorbance scale - 0.2.

The TFN peak was collected in a clean culture tube in approximately 2 ml of solvent to which sufficient NaCl was added to saturate the solution. Two ml of cyclopentane were added to each tube which was then capped and shaken by hand for 15 seconds. The trifluralin, now in the upper cyclopentane layer, was then drawn off into a small culture tube and placed dropwise on a small glass capillary tube in preparation for mass spectrometric analysis. Figure 6 displays representative GPC (first stage cleanup) and HPLC chromatograms for TFN.

Thin Layer Chromatography

Previously collected parathion peaks were dried under N_2, dissolved in 10 drops of $CHCl_3$:MeOH/1:1 and spotted on an Analtech TLC plate which had been previously washed in $CHCl_3$: MeOH/10:90 and activated at 110°C for one hour.

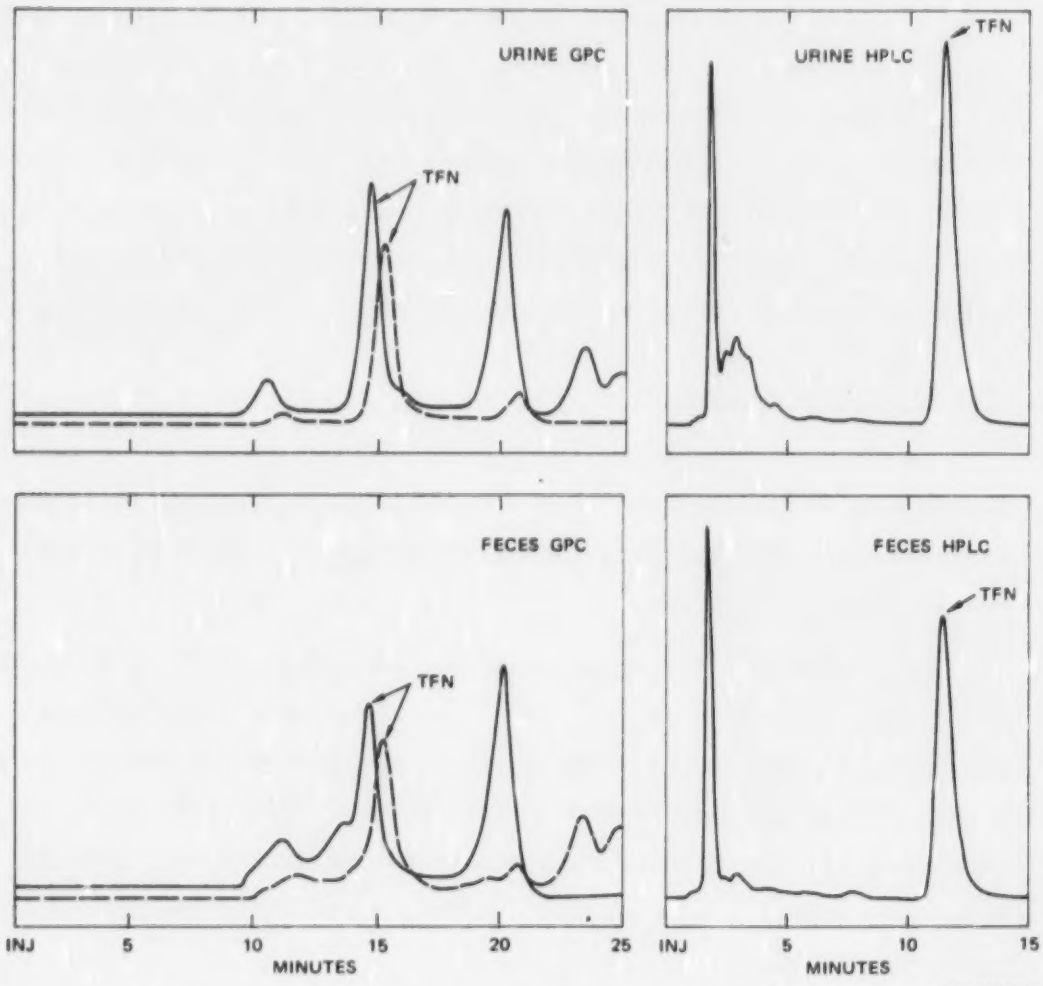
Two dimensional TLC using hexane:acetone/85:15 as the first dimension and benzene as the second gave single spots under U.V. light. These spots were scraped onto a weighing paper, placed into a glass tube having a wad of silanized glass wool for a filter and eluted with ~ 1 ml of $CHCl_3$:MeOH/1:1 into a small glass tube. The sample was then ready for dropwise transfer to a small glass capillary preparatory to mass spectrometric analysis.

FIGURE 6

TRIFLURALIN PURIFICATION
GEL PERMEATION AND REVERSE-PHASE HIGH PRESSURE LIQUID CHROMATOGRAMS
20 μg d_7-TFN SPIKE

GPC: 0.5 ml Injection
Solvent — THF, 1.3 ml/min
——Abs. at 254 nm
– – – Abs. at 280 nm
0.32 O.D. FULL SCALE

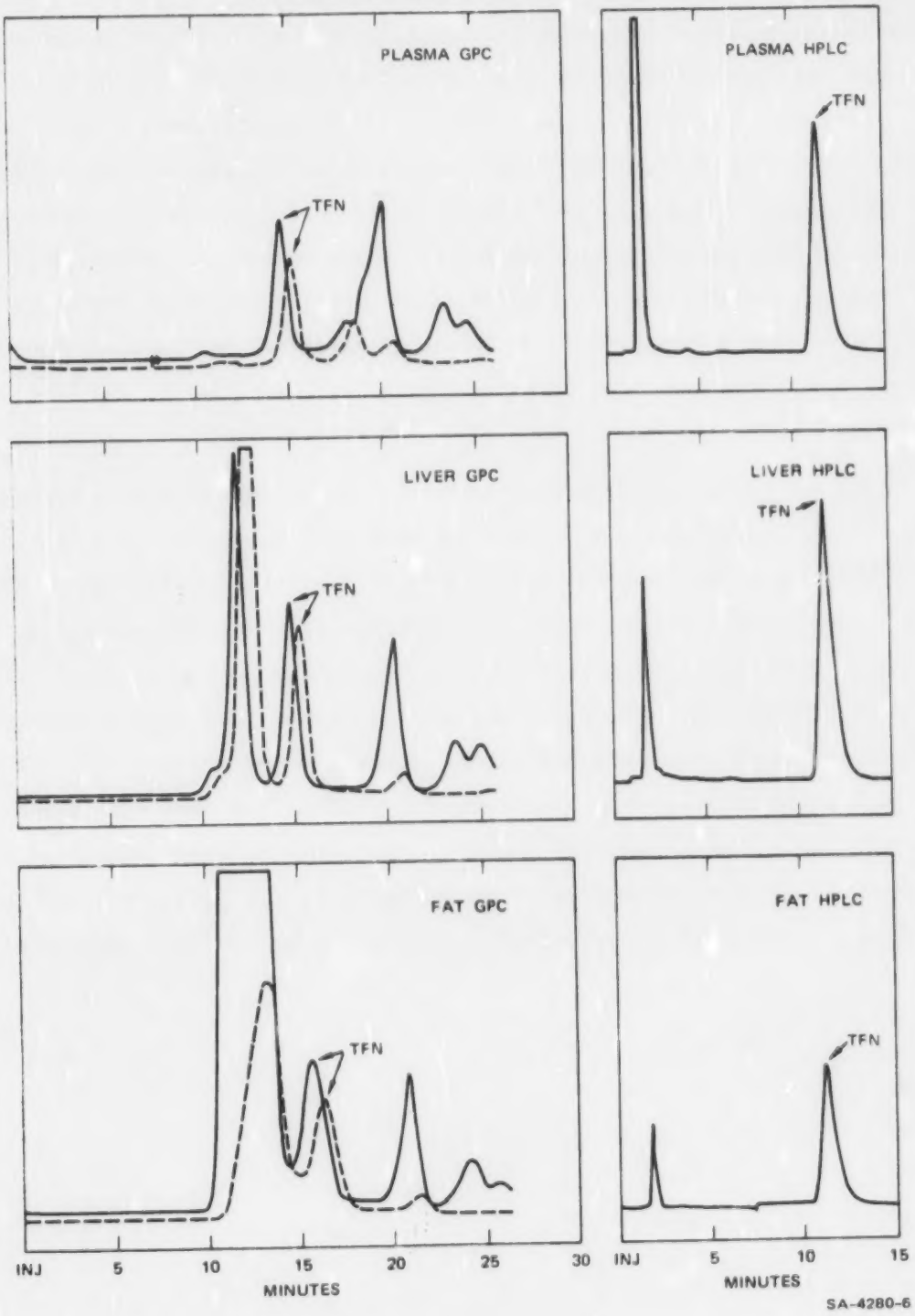
HPLC: 0.5 ml Injection
Solvent — 30:70 H_2O/CH_3OH, 2 ml/min
——Abs. at 254 nm
0.2 O.D. FULL SCALE



SA-4280-5

FIGURE 6A

TRIFLURALIN PURIFICATION (Continued)



Mass Spectrometer Isotope Ratio Measurements

Two sector magnet mass spectrometers were used to obtain the isotope ratio measurements of parathion and trifluralin. A 30° sector instrument was used for the parathion and some of the trifluralin measurements. A 90° instrument was used for the remaining trifluralin measurements. Each machine was equipped with an SRI-designed multipoint field ionization source;[11] the ionizing potential was 1500 volts. The accelerating voltage was scanned repeatedly to span the parent ion region of the material of interest: 288-308 amu for PTN, 332-346 for TFN. Ion counts detected at the electron multiplier were stored in a 1024-channel multichannel analyzer and the counts integrated. When the count rate dropped to background level, the spectrum was recorded and the total counts in each mass tabulated.

The sample was prepared by evaporating the solvent down to a few drops with a gentle flow of dry N_2, which was then placed dropwise on a small glass capillary, allowing the remaining solvent to evaporate in air. The loaded capillary was placed in a quartz solid sample probe and introduced into the spectrometer through a vacuum lock. The sample was cooled to -5° with liquid nitrogen (LN_2) introduced through the back of the probe. The sample was then allowed to warm up and the count accumulation started when the average count rate began to rise above background. PTN came off in the $10\text{-}20^{\circ}C$ range; TFN in the $0\text{-}10^{\circ}C$ range. The count rate was maintained at a reasonable level by occasionally cooling with LN_2. A typical run took 10 minutes. Memory effects were found to be negligible for both materials.

III RESULTS AND DISCUSSION

Cleanup of Extracts from Animal Tissues by High-Pressure Gel Permeation Chromatography

The cleanup of extracts prior to analysis is an essential step in the determination of pesticide and herbicide residues in many animal tissues due to the relatively large amounts of lipophilic substances that may be co-extracted. Conventional methods, summarized recently by Burchfield and Storrs,[7] generally involve partitioning between polar and non-polar organic solvents and/or adsorption on columns of charcoal, Florisil, or other materials. Batchwise or continuous elution yields the pesticide or herbicide in a sufficiently pure state to permit subsequent analysis.

When applied to large numbers of samples, these methods are slow and labor-intensive. Recently, Stalling et al.[15] described a gel permeation procedure for the cleanup of fish extracts that appeared to achieve a fairly clean separation between small pesticide molecules and the bulk of the co-extracted lipids. An even more effective separation could be expected with the aid of highly efficient, microparticulate column packings. A procedure involving high-pressure GPC on columns of μ-Styragel was developed for the present study.

The major advantages of the high-pressure GPC method over conventional methods are:

1. Minimal methods development time is required, since separation depends almost solely on molecular size;

2. Cleanup effectiveness is practically independent of sample size for volumes smaller than 1 ml;

3. The apparatus can be used repeatedly for large numbers of samples without column replacement, since adsorption effects are negligible;

4. A high rate of throughput is maintained, typically 30 min. per sample;

5. Several thousand theoretical plates obtained with microparticulate columns result in high resolution separations.

Following high-pressure GPC, samples can be analyzed immediately by a variety of chromatographic techniques, or they can be further purified for subsequent analysis by mass spectrometry. Low level determinations of pesticides and herbicides in animal tissues by isotope dilution will generally require that additional purification procedures be executed. Both 2-dimensional TLC and reversed-phase HPLC were used in the present study, although other purification techniques, particularly GC, might be advantageous when dealing with volatile compounds.

Analysis of Mass Spectrometric Data

Calculation of Isotope Ratio:

The general formula for calculating the concentration, L (g/g or g/ml sample), of PTN or TFN in a sample is:

$$L = \frac{C_m - C_B}{C_M} \cdot S$$

where C_m = ion counts in minor mass (that of the material originally present in the sample)

C_B = background counts in minor mass (sum of instrument background and of the contribution of trace impurities with the same molecular weight)

C_M = counts in major mass (that of the isotopic multilabeled carrier; background correction is negligible)

S = grams of carrier added per g or ml of sample

For PTN the carrier was d_{10}-PTN, parent ion mass 301 amu (M+9 to M+12 are summed). The material to be assayed was unlabeled PTN, 291 amu.

For TFN the carrier was unlabeled TFN, parent ion mass 335 (M to M+2 are summed). The material to be assayed was d_7-TFN, 342 amu.

Detection Limits:

The ultimate sensitivity of the isotope dilution technique is limited by the background at the minor mass. Total background is the sum of: isotopic background (residual unlabeled material in the carrier), chemical background (impurities at the same mass which carry through from the tissues or are present in the solvents), and instrumental noise (electronic noise and ion scatter).

As was discussed earlier, the high level of residual d_0-TFN in the pure d_7-TFN would have produced an unacceptably high isotopic background at mass 335 if d_7-TFN was used as carrier. Therefore, the reverse dilution assay was performed since the amount of naturally occuring M+7-TFN is nil.

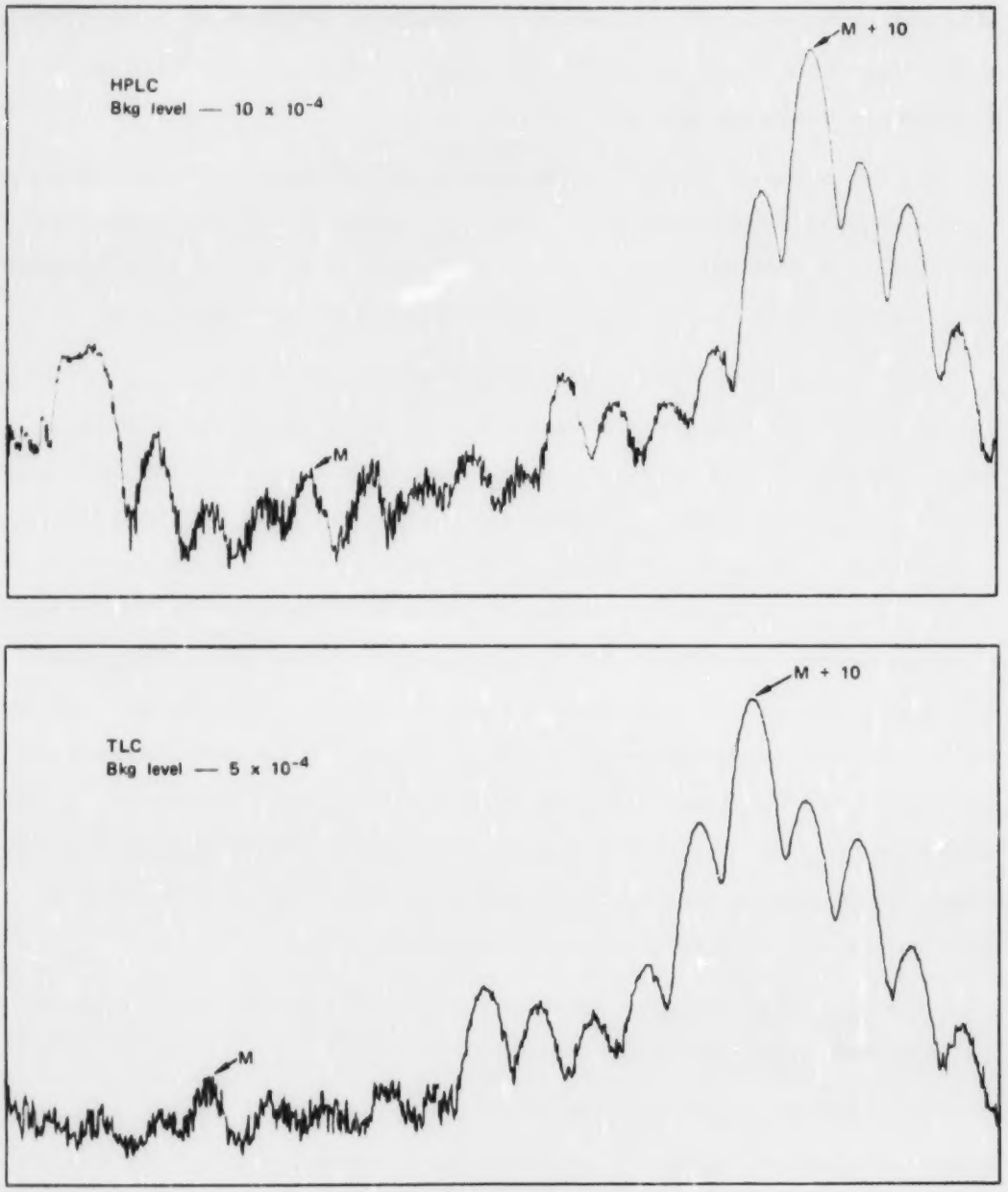
The amount of d_0-PTN in the labeled PTN was at the limit of detection. Inspection of the PTN spectra suggests that the chemical background in the M_0 region is significant, since at each mass there is a definite peak. There are notably accentuated impurity peaks at M+7 and M+8, as well as a peak at M+5 present in the carrier (presumably PTN in which only one ethyl group is deuterated). Figure 7 shows mass scans for a PTN sample purified by reversed-phase HPLC and by 2-dimensional TLC. Since the chemical background is significantly lower following TLC, we selected this method for the second-stage purification. Note that d_0-PTN is clearly present in the TLC sample, but not significantly above background in the HPLC sample. Some chemical background was still present even in the TLC-purified samples, suggesting that a more powerful purification procedure could improve the ultimate sensitivity of detection of PTN.

Figure 8 displays mass scans of PTN plasma samples purified by TLC in which PTN peaks are clearly evident.

For TFN, however, there is an essentially "flat" background in the minor mass region. Figure 9 shows three representative control spectra for the TFN IP series and corresponding samples in which levels of TFN were zero (< 1.1 ng/g), medium (11.5 ± 1.5 ng/g) and high (584 ± 5 ng/g).

FIGURE 7

FIMS SCANS—PARATHION
(LOG DISPLAY)
COMPARISON OF HPLC AND TLC PURIFICATION TECHNIQUES
(2 HR PLASMA—100 μg/kg PTN IP)

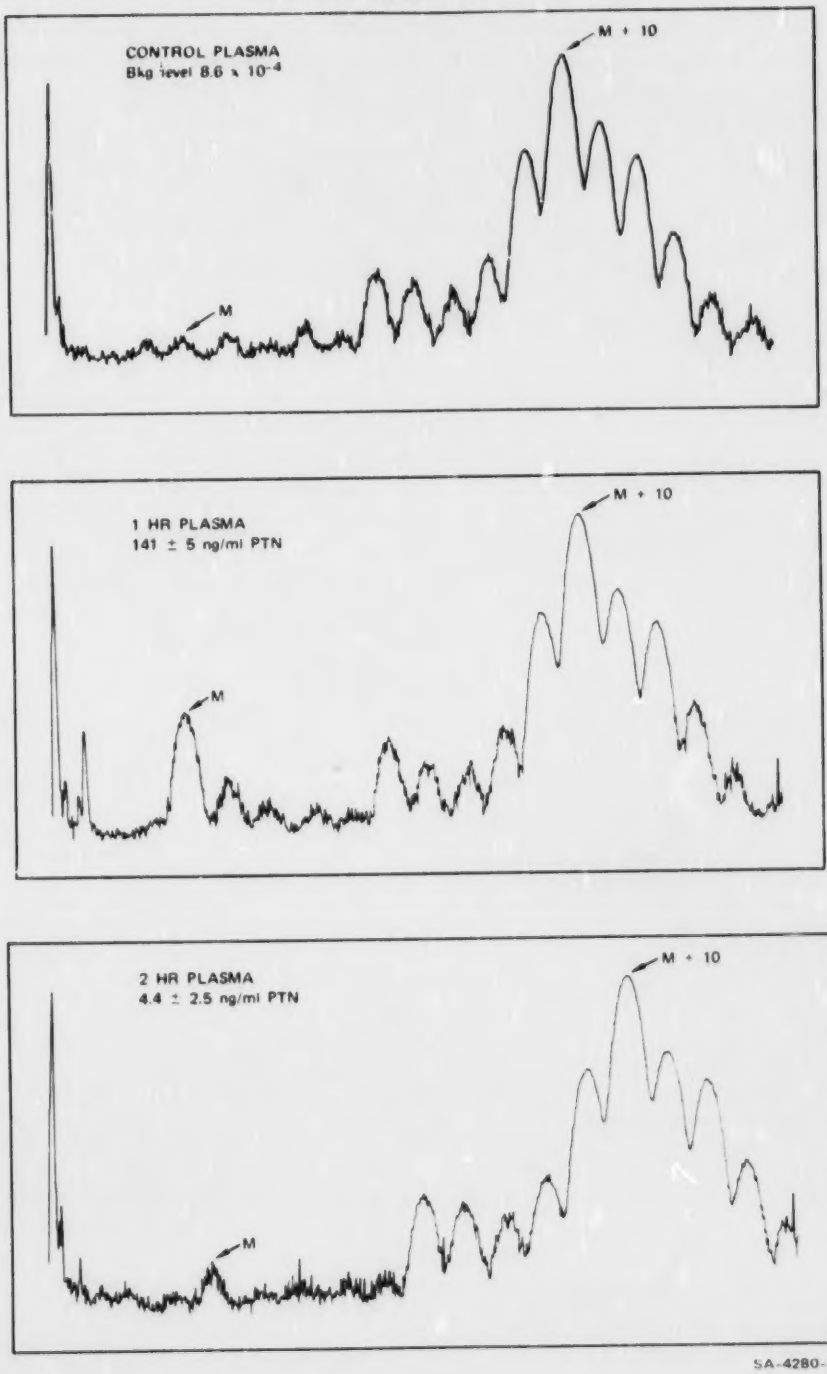


Bkg level: (Average counts in M - 2, M + 1, M + 2) (counts in M + 9 — M + 12.)

SA-4280-7

FIGURE 8

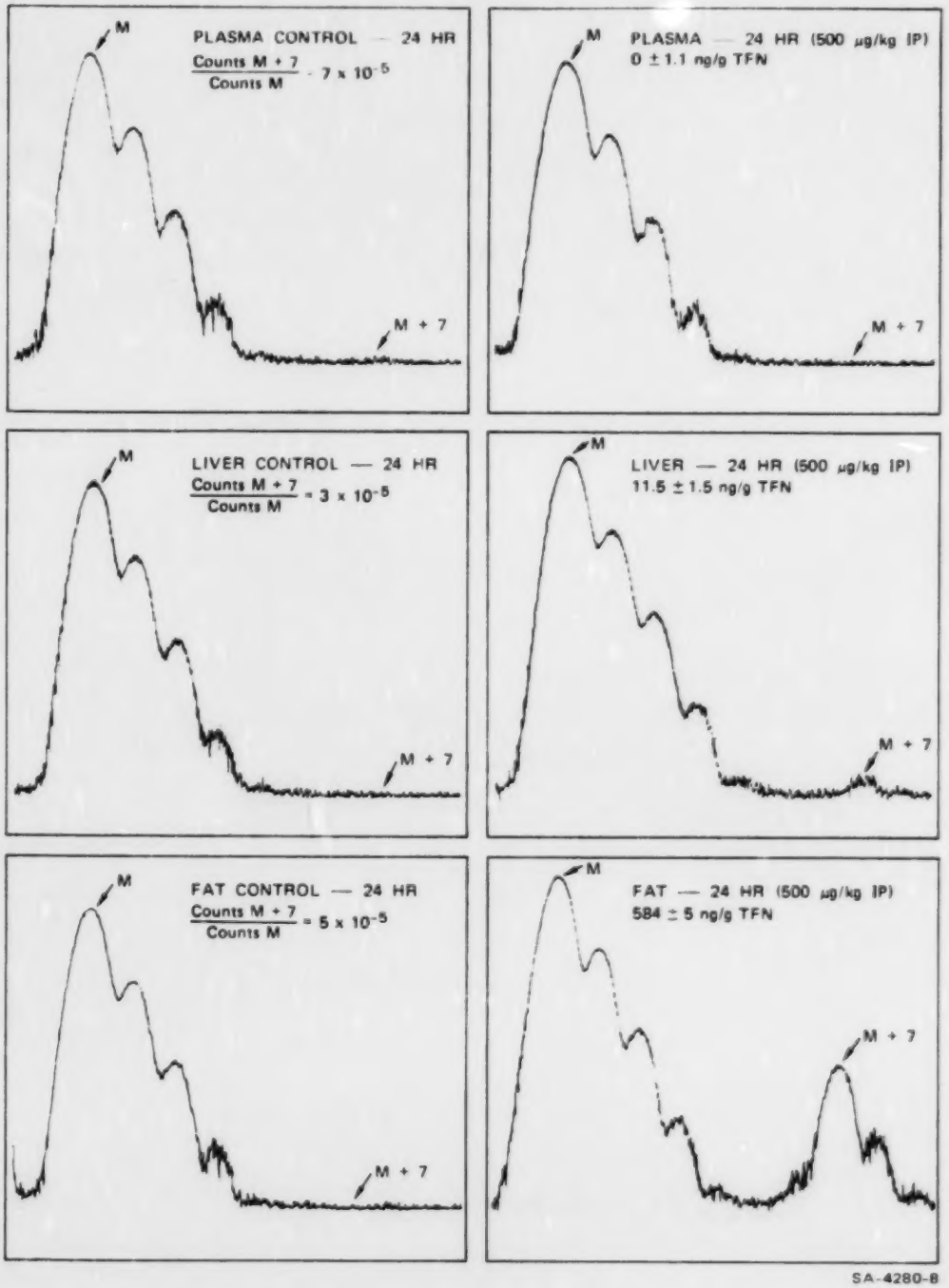
FIMS SCANS—PARATHION
(LOG DISPLAY)
100 μg/kg PTN IP



SA-4280-10

FIGURE 9

FIMS SCANS—TRIFLURALIN IP
(LOG DISPLAY)
REPRESENTATIVE SPECTRA SHOWING UNDETECTABLE, SLIGHT,
AND HIGH d_7-TFN VERSUS CONTROLS


M
PLASMA CONTROL — 24 HR
Counts M + 7 / Counts M = 7 x 10⁻⁵
M + 7
M
PLASMA — 24 HR (500 µg/kg IP)
0 ± 1.1 ng/g TFN
M + 7
M
LIVER CONTROL — 24 HR
Counts M + 7 / Counts M = 3 x 10⁻⁵
M + 7
M
LIVER — 24 HR (500 µg/kg IP)
11.5 ± 1.5 ng/g TFN
M + 7
M
FAT CONTROL — 24 HR
Counts M + 7 / Counts M = 5 x 10⁻⁵
M + 7
M
FAT — 24 HR (500 µg/kg IP)
584 ± 5 ng/g TFN
M + 7

SA-4280-8

Figure 10 shows representative TFN spectra for each tissue in the TFN oral series at the 500 μg/kg feed level. The limit of detection of TFN was determined by the dynamic range attainable with the spectrometer, which is dependent on ion scatter and electronic noise.

Background Correction and Error Analysis:

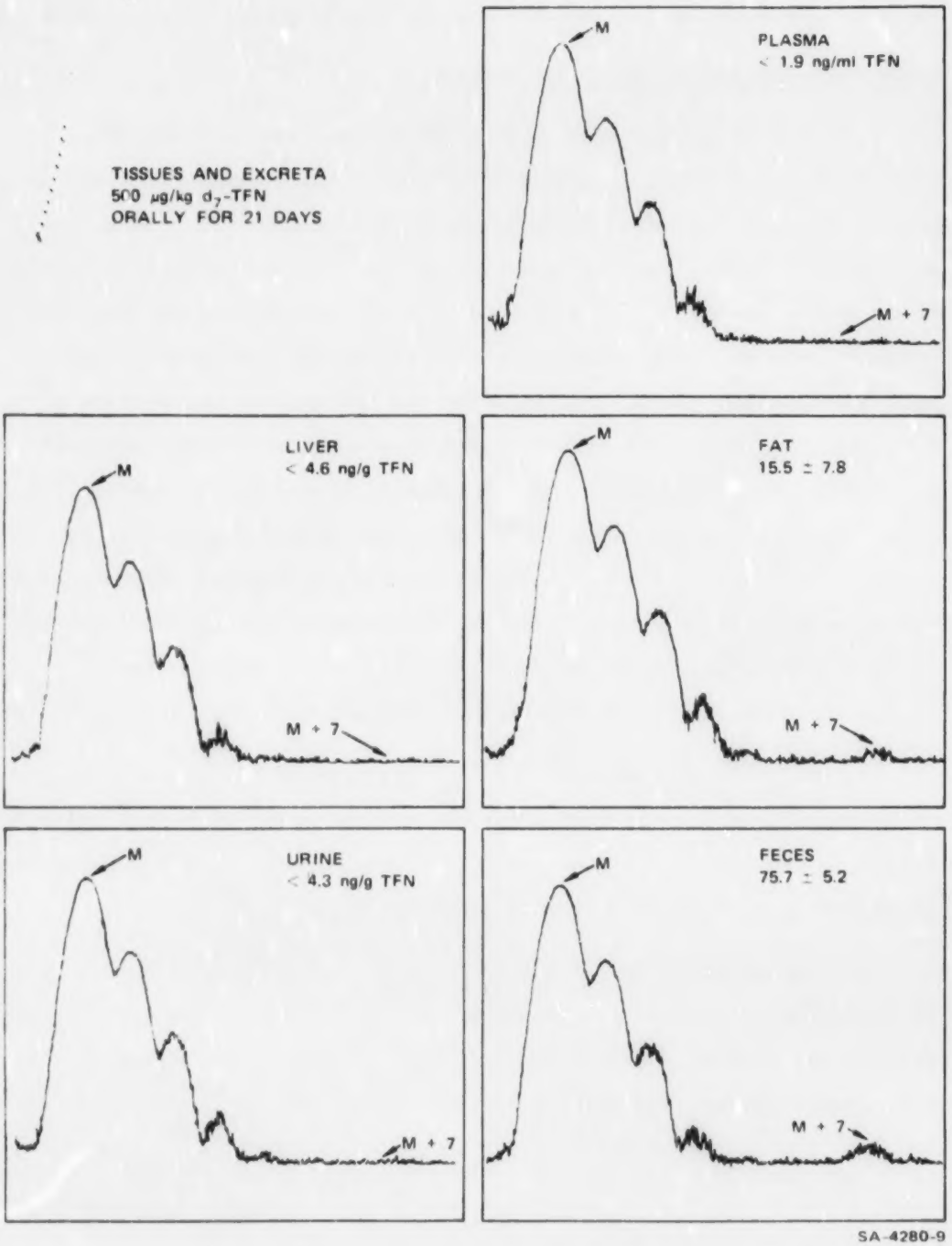
For each individual mass spectrometric analysis, a minor mass background count value, C_B, must be derived. The overall chemical background "profile" in the minor mass region will differ for each material and tissue. Total counts over the entire scan are a function of sample size, which depends on the aggregate yield of the extraction, cleanup and purification steps. Instrumental noise varies according to the instrument tuning and the condition of the ion source. Consequently, in computing the level of material in a sample we cannot simply subtract an average background count figure taken from the control spectra. We must instead obtain an "internal" background value for each scan by inspection of the background level at adjacent mass peaks which bracket the minor mass of interest. Ideally, the chemical background contribution at these mass peaks should be completely absent, with only the unstructured electronic and scattered ion background contributing to the total ion count.

Control samples for each series were used to derive a correction factor by use of which a value of C_B could be calculated for each spectrum according to the scheme described below.

An average background count value, C_{AB}, was obtained for each scan by averaging the counts in the two masses which bracket the minor mass. For PTN the counts in masses 290 and 293 (M-1, M+2) were averaged. For TFN, masses 341 and 344 (M+6, M+9) were used.

FIGURE 10

FIMS SCANS—TRIFLURALIN ORAL
(LOG DISPLAY)



SA-4280-9

An average background correction factor, F_{AV}, was obtained for each control series for a particular material and tissue. For a particular control spectrum,

$$F = \frac{C_m}{C_{AB}}$$

and

$$F_{AV} = \frac{1}{n} \sum_{n} F_n \qquad \text{for n control spectra.}$$

This factor allows us to account for the chemical background relative to that in nearby masses for a particular material and tissue. For example, the background at the minor mass in the PTN red blood cell control samples was on average higher than that in the nearby masses ($F_{AV} = 1.3$); in PTN plasma, however, the minor mass background was lower ($F_{AV} = 0.65$). Using the background correction factor we can thus "internally" compute a value for C_B for each scan which properly reflects the background profile in each tissue, and which accounts for sample size and instrument noise variations reflected in the absolute magnitude of C_{AB}:

$$C_B = F_{AV} \cdot C_{AB} \quad \text{(for each spectrum in a particular material/tissue series)}$$

The standard deviation in the value for F_{AV}, σ_F, was also computed for each control set. This reflects the variations in chemical background level due to variations in the extraction and purification procedures.

Using the equation

$$L = \frac{C_m - C_B}{C_M} \cdot S$$

we may then calculate the level of PTN or TFN in a particular sample in ng/g or ng/ml.

The variance in a particular measurement depends on the variance in F_{AV}, the statistical variance in the minor mass and major mass count values, and on the volumetric error in the carrier spike. Counting error in the major mass is small relative to that in the minor mass and can be neglected. The standard deviation in the counts at the minor mass, σ_m, was computed by the formula,

$$\sigma_m = \left[C_m + C_{AB} + (\sigma_F F_{AV})^2 \right]^{1/2}$$

which expresses the variance due to counting error in the minor mass and due to variance in chemical background in the controls.

Standard deviation in the measured concentration of PTN or TFN in a sample, σ_L, is then expressed as

$$\sigma_L = \frac{\sigma_m}{C_m - C_B} \cdot L$$

Each value for the level of PTN or TFN in the tables in the following section is expressed as $L \pm \sigma_L$; σ_L does not include the volumetric error in the spike, which is of the order of 1-2 percent.

In some scans, the number of counts in the minor mass, C_m, was less than or equal to C_B, which corresponds to a "negative sample". This impossibility arises because of the uncertainties in F_{AV}, computed from the controls. In those instances, the measured level is expressed as $0 \pm \sigma'_L$ where

$$\sigma'_L = \frac{\sigma_m}{C_m} \cdot S, \ \sigma'_L \text{ being the limit of detection}$$

There are also many marginal cases near the detection limit--below 5 ng/g or so--where $L \leq \sigma_L$.

In those series where significant amounts of material were found, e.g., liver and fat TFN-IP, a grand average was computed for each set of measurements (3 rats replicate spectra). The error limits placed on these values reflect both animal and measurement variances, the former being predominant. Average detection limits were computed for those sets where the material was not found to be present.

Determination of PTN in Rat Tissue by Isotope Dilution

The measured levels of PTN for the 100 ug/kg IP regimen are displayed in Table 4, and the results summarized in Table 5.* Replicate scans were obtained for some samples. Detection limits differed among the tissues because of differences in chemical background. Liver and brain were the cleanest samples, as traces of PTN could be identified at the 1-2 ng/g level. Chemical background was higher for fat, plasma and red blood cells; PTN below the 5 ng/g level could be identified in only one sample (2 hr plasma, rat #2).

Because the PTN levels were near or below the detection limits in the 2 hr samples (with the exception of one anomalous fat sample) we analyzed for PTN in only a few samples collected at later times. No PTN was found in these samples.

A number of methods have been applied for the determination of PTN in animal tissues.[7] Gas chromatography with alkali flame ionization detection or electron capture detection appears to be among the most sensitive analytical procedures currently being used for this compound. Quantitation of PTN down to about 50 ng/ml of plasma is feasible by the addition of methyl parathion as an internal standard.

With isotope dilution analysis and FIMS, quantities of PTN down to about 2 ppb were measured in the present study. The sensitivity was limited primarily by the chemical background in the mass spectrum, following purification by 2-dimensional TLC or reversed-phase HPLC. Similar impurities were observed in the mass spectrum of PTN after both chromatographic techniques had been applied. This observation suggests

*Raw count data are compiled in the Appendix

TABLE 4

MEASURED PARATHION LEVELS IN RATS (ng/g or ng/ml)
SINGLE DOSE, 100 μg/kg PTN IP; RATS SACRIFICED AT 1 AND 2 HR[+]
(3 RATS; MULTIPLE MASS SPECTROMETER RUNS)

		1 HR		2 HR	
LIVER	Rat #1	0 ± 1.2	0 ± 1.5	3.7 ± 1.1	0.3 ± 1.0
	#2	0.2 ± 1.2	0 ± 1.4	1.8 ± 1.5	0 ± 1.3
	#3	0 ± 1.2		2.5 ± 1.2	4.7 ± 1.4
FAT	Rat #1	0.2 ± 2.3	1.7 ± 1.9	0 ± 3.0	
	#2	0 ± 3.9		170[*] ± 7	152[*] ± 5
	#3	0 ± 3.1	0 ± 6.0	9.6 ± 2.8	
PLASMA	Rat #1	27.0 ± 3.9		0[+] ± 10.1	
	#2	141.0 ± 5.0		4.4 ± 2.5	
	#3	0 ± 4.3		1.3 ± 4.1	
BRAIN	Rat #1	3.9 ± 1.2 2.7 ± 1.1	3.6 ± 1.2 2.0 ± 1.3	0.2 ± 0.7	5.0 ± 1.6
	#2	2.1 ± 1.2 2.6 ± 1.3	1.9 ± 0.9	9.4 ± 1.4 10.0 ± 1.6	9.3 ± 1.0
	#3	7.1 ± 1.2	14.2 ± 1.2	1.2 ± 1.9	1.0 ± 0.6
RBC	Rat #1	0[+] ± 13.5	0[+] ± 10.5	0 ± 4.2	
	#2	0 ± 3.6	0 ± 3.0	0 ± 4.8	0 ± 4.1
	#3	0 ± 3.5	0 ± 3.8	0 ± 4.2	0 ± 4.6

[*]Possible accidental injection into fat; see Discussion

[+]High background contamination

Table 5

SUMMARY

PARATHION IP

Single dose, 100 µg/kg IP; rats sacrificed at 1 and 2 hr post-injection.

LIVER: 1 hr-- None detectable, < 1.5 ng/g (average 3 rats)
2 hr-- Trace, 2.2 ± 1.2 ng/g (average 3 rats)

FAT: 1 hr-- None detectable, < 4 ng/g (average 3 rats)
2 hr-- None detectable, < 3 ng/g (rat #1)
161 ± 9 ng/g (rat #2) Possible improper injection into fat.
9.6 ± 2.8 ng/g (rat #3)

PLASMA: 1 hr--27.0 ± 3.9 ng/ml (rat #1)
141 ± 5 ng/ml (rat #2)
None detectable, < 4 ng/ml (rat #3)
2 hr--None detectable, < 6 ng/ml (average 2 rats)
Trace, 4.4 ± 2.5 ng/ml (1 rat)

BRAIN: 1 hr--10.6 ± 0.8 ng/g (1 rat)
Trace, 2.6 ± 0.5 ng/g (average 2 rats)
2 hr--9.6 ± 0.8 ng/g (1 rat)
Trace, 1.8 ± 1.1 ng/g (average 2 rats)

RED BLOOD CELLS: 1 and 2 hr--None detectable, < 4 ng/g average

the possibility that a solvent common to both methods was the source of the impurities. Methanol was used both for elution of PTN from silica gel and as a solvent in reversed-phase HPLC.

It seems probable that higher sensitivities of detection might have been achieved in the case of PTN by the use of gas chromatography for purification. Our laboratory is not, at present, equipped for routine, preparative gas chromatographic separations. We intend, however, to develop this capability during the coming year for pesticide applications.

The results of the investigation of PTN levels in different tissues of rats following IP injection at a dose of 100 μg/kg indicate that the pesticide disappears rapidly from plasma. Low but significant levels were found in the brain tissue of all rats sacrificed 1 and 2 hr post-injection. One of three rats killed at 2 hr post-injection appeared to have low levels of PTN in both liver and fat; evidence supporting the presence of PTN in the liver and fat of the other two animals was not compelling (see below).

The rapid rate of disappearance of PTN from rat plasma is consistent with the findings of Vukovich *et al*.[16] Following intravenous injection of rats with 4 mg/kg of parathion, these investigators determined the PTN levels in plasma for times up to 90 minutes. An average half-life of about 50 minutes for PTN in rat plasma can be estimated from their data. This finding seems to agree with our own results, since it implies that the PTN concentration would have fallen to an essentially undetectable level by about four hours post-injection. Unambiguous determinations of PTN were made in two rats at 1 hr and in one rat at 2 hr, but not in the samples collected subsequently.

The presence of PTN in brain at 1 and 2 hr post-injection is consistent with rapid equilibration of the pesticide between that organ and plasma. This is not surprising, since the brain is well-endowed with vascular tissue and readily absorbs lipophilic substances.[17] A similar

result would be expected for liver, since it also is a well-perfused organ, but the experimental findings indicate that the levels of PTN in liver were negligible in most cases. It is probable that the high metabolic activity of liver is responsible for the virtual absence of unchanged PTN in that organ.

Other workers have established a complex metabolic pathway for PTN in mammalian systems.[18-25] In cases in which heavy doses of PTN were ingested, sufficient to cause death, it has been possible to demonstrate high levels of PTN in human liver at autopsy and in rat liver.[26-28] In these unusual cases, however, it is likely that the normal metabolic pathways, which depend on an O_2 supply, were essentially inoperative.

It is somewhat surprising that PTN was not detected in any of the 1 hr fat samples, when it is still present at significant levels in plasma. No positive explanation can be given for the absence of PTN in most of the 1 and 2 hr fat samples, although slow uptake due to the low degree of vascularization of adipose tissue, together with the rapid disappearance of PTN due to metabolism and reaction, is probably responsible. One rat sacrificed at 2 hr did exhibit a very high level of PTN in fat. This case seems, however, to be anomalous, and could have been caused by accidental injection of the PTN directly into the fat tissue, in which it would be expected to remain as a deposit for an extended period of time.

The disappearance of PTN in rats is evidently quite rapid, and the compound seems to be distributed extensively among the well-perfused tissues and organs of the animal. Storage of PTN in adipose tissue, however, does not appear to be significant, at least at low dose levels. This is encouraging from the viewpoint of exposure of humans or animals to PTN, since it implies that gradual build-up of the compound to toxic levels due to repeated exposures is unlikely.

Although PTN has been found at generally low levels in tissues following IP injection, it is quite probable that metabolites of PTN would be present in the same tissues at much higher quantities. A sensitive analytical procedure that assays for one or more metabolites of PTN might employ similar methods to those used here. Such a procedure could conceivably detect residues of PTN metabolites for extended periods of time after exposure.

Determination of TFN in Rat Tissues by Isotope Dilution

The measured TFN levels for the 500 μg/kg IP regimen are displayed in Table 6 and the results summarized in Table 7.* All samples were run in duplicate. In the liver series, with TFN concentrations in the 5-50 ng/g range, the agreement between duplicate runs is good, in all cases within the standard deviation limits calculated for each value. Average relative deviation from the mean is ~7%. In the high level fat series, agreement is excellent; average deviation from the mean is ~2%. (There is one exception, 4 hr fat, rat #1, in which the discrepancy is 22%; this may be due to a mistake in recording the counts).

Animal variation in measured TFN value is, however, considerable--a predictable result.

One rat in the 8 hr set had no measurable TFN in liver or fat, most probably because that animal was mistakenly not injected with TFN. This rat was omitted when calculating the averages.

The measured TFN levels for the daily oral administration of TFN at 3 levels are displayed in Table 8 and summarized in Table 9.* TFN was found in only a few samples. Because of time constraints, and because the reproducibility of the mass spectrometric determinations was already established by the TFN IP measurements, only those samples in which TFN was present were run in duplicate.

*Raw count data are compiled in the Appendix

TABLE 6

MEASURED TRIFLURALIN LEVELS IN RATS (ng/g or ng/ml)-IP ADMINISTRATION
SINGLE DOSE, 500 µg/kg TFN IP; RAT SACRIFICED AT 4, 8, 24, 48, 72 HOURS
(3 RATS; MASS SPECTROMETER RUNS IN DUPLICATE)

		4 HR	8 HR	24 HR	48 HR	72 HR
LIVER	Rat #1	48.0 ± 5.5	0* ± 2.1	31.4 ± 4.7	0.8 ± 1.4	2.2 ± 1.1
		37.4 ± 5.5	0* ± 1.7	23.2 ± 1.6	1.6 ± 1.2	0 ± 1.6
	Rat #2	53.2 ± 6.1	23.4 ± 2.8	12.4 ± 4.7	7.9 ± 1.3	0.2 ± 1.2
		42.0 ± 7.0	23.2 ± 2.2	11.5 ± 1.5	6.3 ± 1.5	0 ± 1.8
	Rat #3	60.2 ± 6.0	8.3 ± 1.8	10.3 ± 5.1	8.1 ± 1.4	2.8 ± 1.6
		68.4 ± 7.8	7.9 ± 2.2	9.1 ± 1.4	7.5 ± 1.6	2.1 ± 1.3
FAT	Rat #1	728 ± 19	2.2* ± 3.5	584 ± 5	847 ± 8	1935 ± 8
		466 ± 16	0* ± 3.7	578 ± 6	867 ± 16	1891 ± 9
	Rat #2	273 ± 8	261 ± 7	1833 ± 10	624 ± 5	81.3 ± 20
		253 ± 11	251 ± 5	1621 ± 22	626 ± 9	86.3 ± 3
	Rat #3	414 ± 8	697 ± 19	2386 ± 13	1282 ± 10	725 ± 7
		369 ± 11	632 ± 16	2343 ± 19	1302 ± 17	721 ± 9
PLASMA	Rat #1	4.7 ± 2.8	0* ± 3.2	0 ± 1.1	0 ± 3.0	0 ± 1.0
		0 ± 2.7	0* ± 4.0	0 ± 0.9	0 ± 2.8	0 ± 1.0
	Rat #2	0 ± 2.6	5.9 ± 4.0	0 ± 0.8	0 ± 0.7	0 ± 0.8
		0 ± 3.3	0 ± 1.2	0 ± 0.9	0 ± 2.7	0 ± 0.9
	Rat #3	0 ± 2.9	0 ± 2.8	0 ± 1.2	0 ± 1.1	0 ± 0.8
		0 ± 4.0	0 ± 2.6	0 ± 1.6	0 ± 2.7	0 ± 1.0
URINE	Rat #1			0.9 ± 2.1	0.5 ± 0.6	0 ± 1.0
				2.6 ± 2.1	0.6 ± 0.8	0.9 ± 0.8
	Rat #2			2.3 ± 2.4	0.1 ± 0.8	1.0 ± 0.7
				5.9 ± 2.1	0 ± 0.8	1.0 ± 0.7
	Rat #3			0.1 ± 2.1	0.7 ± 1.0	0 ± 0.6
				1.1 ± 2.7	0.7 ± 0.9	0 ± 0.7
FECES	Rat #1			5.5 ± 3.1	0.5 ± 0.7	0 ± 4.0
				0 ± 2.7	0 ± 0.8	3.2 ± 2.3
	Rat #2			0 ± 1.3	0 ± 0.7	0.5 ± 1.2
				0 ± 3.4	0 ± 0.9	
	Rat #3			0.6 ± 2.9	0.7 ± 0.8	1.3 ± 1.9
				0 ± 1.1	0 ± 0.6	9.8 ± 7.6

* Rat apparently not injected with TFN

TABLE 7

SUMMARY

TRIFLURALIN IP

Single dose, 500 µg/kg IP; rats sacrificed at 4, 8, 24, 48, 72 hr post-injection.

LIVER:
4 hr--51.5 ± 9.6 ng/g (average 3 rats)
8 hr--15.7 ± 7.7 ng/g (average 2 rats)
24 hr--16.3 ± 9.7 ng/g (average 3 rats)
48 hr-- 5.4 ± 3.0 ng/g (average 3 rats)
72 hr-- None detectable, < 2.4 ng/g (average 2 rats)
Trace, 2.5 ± 1.0 ng/g (1 rat)

FAT:
4 hr-- 417 ± 138 ng/g (average 3 rats)
8 hr-- 461 ± 205 ng/g (average 2 rats)
24 hr--1558 ± 737 ng/g (average 3 rats)
48 hr-- 925 ± 274 ng/g (average 3 rats)
72 hr-- 907 ± 750 ng/g (average 3 rats)

PLASMA: None detectable, < 2.8 ng/ml average

URINE:
24 hr-- Trace, 2.1 ± 1.7 ng/ml (average 3 rats)
48 and 72 hr-- None detectable, < 1.8 ng/ml average

FECES: None detectable, < 1.6 ng/g average

TABLE 8

MEASURED TRIFLURALIN LEVELS IN RATS (ng/g or ng/ml) - ORAL REGIMEN

TFN ADMINISTERED ORALLY FOR 21 DAYS AT 3 DOSE LEVELS

(3 RATS; SOME MASS SPECTROMETER RUNS IN DUPLICATE)

	LIVER	FAT	PLASMA	URINE			FECES		
				7 DAY	14 DAY	21 DAY	7 DAY	14 DAY	21 DAY
20 µg/kg Rat #1	0 ± 2.9	1.6 ± 1.6	0 ± 1.8	5.1 ± 6.0	0 ± 1.4	0 ± 2.7	0 ± 8.0	0 ± 2.9	0 ± 2.7
#2	0 ± 3.7	0 ± 2.3	0.6 ± 2.1	0 ± 5.9	0 ± 1.2	0 ± 3.7	0 ± 3.8	0 ± 1.8	0 ± 1.9
#3	0 ± 3.0	4.9 ± 3.9	2.7 ± 2.1	0 ± 4.3	0.4 ± 1.6	0 ± 2.4	0 ± 4.8	0 ± 2.2	0 ± 3.0
100 µg/kg Rat #1	0 ± 2.7	7.1 ± 2.3 5.8 ± 2.1	0 ± 2.5	0 ± 1.6	0.4 ± 1.2	0.4 ± 1.3	0 ± 2.2	0 ± 4.7	4.7 ± 3.5
#2	0 ± 2.9	3.4 ± 3.2 1.6 ± 2.5	0 ± 1.8	0 ± 2.0	0 ± 1.5	0 ± 1.5	0 ± 3.3	0 ± 2.1	0 ± 2.5
#3	0 ± 2.9	25.8 ± 10.1 11.2 ± 3.3	0 ± 2.1	0 ± 1.6	0.2 ± 0.9	0.6 ± 3.4	0.5 ± 2.0	0 ± 2.0	0.3 ± 2.7
500 µg/kg Rat #1	0 + ± 20.2	17.0 ± 8.4 15.5 ± 2.8	0.2 ± 1.2	0 ± 2.5	1.8 ± 1.6	2.0 ± 2.3	6.2 ± 3.6 6.2 ± 2.4	188 ± 9 195 ± 8	35.3 ± 3.7 23.6 ± 9.2
#2	0 ± 3.6	5.5 ± 1.8 1.5 ± 3.8	1.1 ± 2.1	7.9 * ± 4.5	0.1 ± 1.7	1.4 ± 2.3	1.5 ± 2.2 3.1 ± 5.1	7.5 ± 2.5	46.9 ± 8.1 75.7 ± 5.2
#3	0 ± 3.9	8.5 ± 3.3 9.8 ± 2.2	0 ± 1.9	0.5 ± 2.6	1.2 ± 1.5	0 ± 1.7	78.1 ± 20.0 73.1 ± 5.2	0 ± 2.1 5.2 ± 3.1	3.6 ± 3.3

* High background contamination

\+ Low total counts

TABLE 9

SUMMARY

TRIFLURALIN ORAL

Daily oral dose (by stomach tube) for 21 days at 20, 100 and 500 µg/kg levels. Rats sacrificed at 21 days. 24 hr urine and feces collected at 7, 14, and 21 days.

LIVER: None detectable at any level, < 3.2 ng/g average

FAT: 20 µg/kg: None detectable, < 3.2 ng/g (average 2 rats)
Trace, 4.9 ± 3.9 ng/g (1 rat)

100 µg/kg: 9.1 ± 7.1 ng/g (average 3 rats)

500 µg/kg: 9.6 ± 5.5 ng/g (average 3 rats)

PLASMA: None detectable at any level, < 1.9 ng/ml average

URINE: None detectable at any level/time, < 2.3 ng/ml average

FECES: 20 and 100 µg/kg: None detectable at any time, < 3.1 ng/g average

500 µg/kg: rat #1--6.2 ± 2.2 ng/g (7 days); 191 ± 6 ng/g (14 days); 29.5 ± 5.0 ng/g (21 days)

rat #2--< 3.5 ng/g (7 days); 7.5 ± 2.5 ng/g (14 days); 61.3 ± 4.8 ng/g (21 days)

rat #3--73.4 ± 5.2 ng/g (7 days); 2.6 ± 1.9 ng/g (14 days); 3.6 ± 3.3 ng/g (21 days)

Conventional techniques for the detection or analysis of TFN include TLC and GC. The latter can be made quantitative with the addition of diisobutyl phthalate as an internal standard.[29] The limit of sensitivity of these methods, when applied to animal tissues, has not been reported to our knowledge, but is probably similar to that of PTN (50 ng/g). A novel method involving spark source mass spectrometry has been described for the analysis of TFN in soils.[30] This method takes advantage of the relatively low fluorine background in soils; a sensitivity limit of about 1 ppb is claimed. This method, however, is non-specific.

A sensitivity of the order of 1 ppb was achieved in the present study using isotope dilution analysis and FIMS. Following cleanup by high-pressure GPC and purification by reversed-phase HPLC, background impurities in TFN extracts were below the point of detection. Scattered ion noise in the mass spectrometer set the limit of detectability at about 1 ppb in animal tissues. Identification of trifluralin is, moreover, unambiguous in this method.

The scattered ion background in TFN samples was slightly higher than has been found in some other applications of the method.[11] This was probably due to an instrumental alteration that preceded the analyses of TFN. A retarding lens that is normally situated near the detector had been removed to allow some other instrumental modifications to be made. In prior applications, this lens had reduced the scattered ion background by a factor of 5 without significantly reducing the signal. An improvement in the signal/noise ratio by this factor would allow analyses of pesticides and herbicides to be performed in the subnanogram range.

Following IP injection of TFN (500 μg/kg), significant levels were found in liver for at least 48 hr post-injection. High levels of TFN were found in fat at 72 hr post-injection, the longest time that was studied. No detectable unchanged TFN was present in plasma or feces at any time up to 72 hours. Trace levels of TFN appeared in urine during the first 24 hours, but not at later times.

For pharmacokinetic purposes, it is generally assumed that the well-perfused tissues, e.g., liver, lung, heart, spleen, and brain, constitute together with plasma a single "compartment" within which a drug or other foreign compound is equilibrated rapidly according to its relative affinities for the different tissues in the compartment (see for example, discussions in refs. 31-33). Poorly perfused tissues, such as muscle, bone, and fat depots, constitute a second "compartment". Interactions between these compartments, together with metabolism and excretion, define the kinetics of the compound in question.

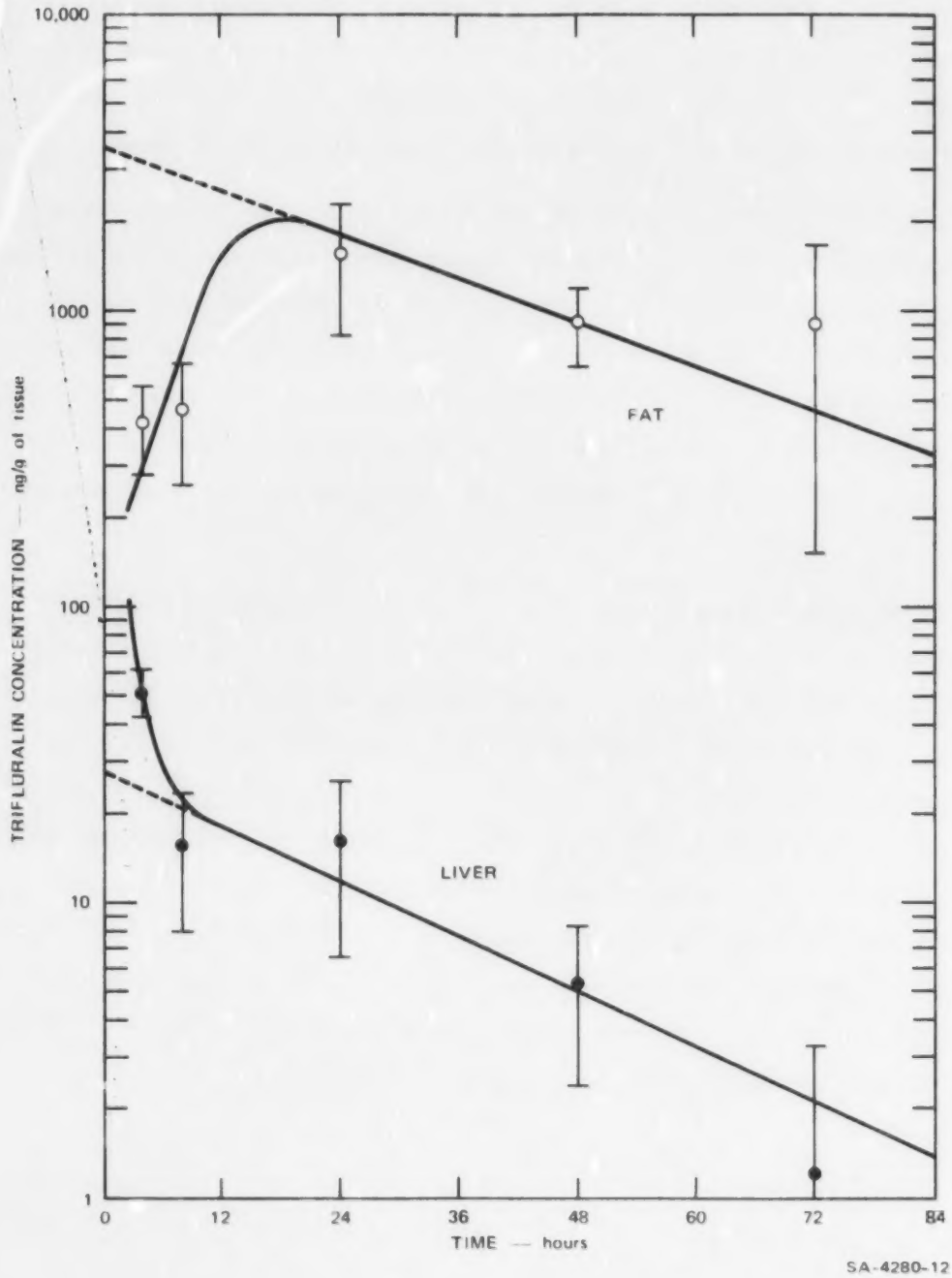
The compartmental hypothesis is an over-simplification, since equilibration among the different tissues of a compartment is not instantaneous. Nevertheless, surprisingly good agreement among different tissues has been found in the kinetics of at least one compound[33] when the levels were determined in several different organs of rat.

To the extent that the liver may be regarded as representative of perfused tissues in general, and fat as representative of the less well-perfused tissues, the terminal half-life of TFN in the rat can be estimated (Fig. 11). For the two tissues, the estimated terminal half-lives are 20 and 25 hours, respectively. Considering the paucity of data, the assumptions involved in the model, and the fact that the lines were drawn visually rather than by least-squares, the agreement between these values, although predicted, is perhaps fortuitous. These results, in essence, provide the basic information needed to undertake a more thorough study of the persistence, kinetics, and distribution of TFN in rats.

The major identified metabolites of TFN in rats result from dealkylation of the N-propyl group and reduction of the nitro group(s).[34-36] Since one of the two propyl groups of the TFN injected into the rats was labeled with deuterium, it is probable that the rate of metabolism of the injected compound via N-dealkylation is not exactly equal to the rate of metabolism of unlabeled TFN. The half-lives of the two isotopic variants should not

FIGURE 11

TRIFLURALIN CONCENTRATIONS IN ADIPOSE TISSUE AND LIVER OF ADULT MALE SPRAGUE-DAWLEY RATS FOLLOWING INTRAPERITONEAL INJECTION WITH TRIFLURALIN. DOSE: 500 μg/kg. EACH POINT REPRESENTS THE AVERAGE VALUE OBTAINED FROM 3 RATS. ERROR BARS ARE ESTIMATED STANDARD DEVIATIONS.



differ substantially, however, because (1) only one of the two propyl groups was labeled with deuterium, and (2) N-dealkylation accounts for a relatively minor fraction of the total metabolites produced in rats.[34]

Following chronic oral administration of TFN at three different levels to rats (20 μg/kg, 100 μg/kg, and 500 μg/kg) for 21 days, the compound was detected both in fat and feces at the higher dose levels, but was not found in liver, plasma, or urine. The levels found in fat were low in comparison to the results of IP injection, and the levels in feces were also low in comparison to the quantities administered.

A similar study was carried out several years ago by Emmerson and Anderson,[34] in which rats were chronically fed high doses of TFN (100 mg/kg), radiolabeled with ^{14}C. These authors also observed very little unchanged TFN in the feces (< 8% of an oral dose), but found that approximately 70% of the radioactivity was eliminated as metabolites in feces, the remainder being excreted as metabolites in urine. They observed only low levels of TFN in fat, following chronic oral toxicity studies.

The results of our study are seen to be in substantial agreement with those of Emmerson and Anderson.[34] The latter authors postulated that TFN is poorly absorbed from the gut and is extensively metabolized within the intestines, probably by micro-organisms. Such an hypothesis provides a reasonable explanation for the present findings. The oral route of TFN administration evidently results in little systemic absorption of the unchanged herbicide. However, the IP studies suggest that, once absorbed, the compound remains in tissues for rather lengthy periods of time. A study of the long-term persistence of TFN in these and other tissues of rat could be undertaken readily using the techniques developed in this research program.

IV DETERMINATION OF THE CONCENTRATION OF PTN AND MePTN IN AEROSOLS

The concentrations of parathion and methyl parathion in the inhalation exposure chambers in the Toxicology Department of SRI[37] were measured by an isotope dilution technique similar to that used to measure the concentration of explosive vapors in air.[38] There are four inhalation chambers in series; the material was dispersed in an aerosol by a nebulizer (methyl parathion 80% in xylene) and the airflows adjusted to reduce the concentration by a factor of two in each succeeding chamber in the line. Running conditions were selected to match those of previous toxicology experiments with these materials.

Experimental

The chamber air was sampled with 50 ml glass bulbs with Teflon stopcocks. The stopcock was removed and 0.5 ml of a THF solution of the isotopic diluent added through a flexible Teflon tube attached to a syringe. 136 μg of d_{10}-PTN (determined by absorbance) or 100 μg of d_6-MePTN (determined gravimetrically) were loaded into the sample bulb. The solvent was evaporated by a stream of dry N_2 and the stopcock replaced. The bulb was then immersed in LN_2 and evacuated on a small vacuum line.

The evacuated bulb was attached to the sampling port of the inhalation chamber, allowed to equilibrate for 5 minutes, the stopcock opened briefly to sample the air, and then closed.

After a 10 min. wait to allow the sampled air to equilibrate with the multilabeled carrier, the stopcock was removed and the bulb interior washed out with two 1 ml portions of spectrograde THF, introduced and withdrawn with a syringe equipped with a flexible Teflon tube. The solvent was evaporated off and the sample transferred to a small glass capillary for mass spectrometer ratio determination in the previously described manner. No prior purification of the samples was attempted.

The chamber air flows were set to give nominal concentrations of 250 mg/m^3 PTN and 380 mg/m^3 MePTN in chamber #1. These values were based on glc determinations made many months previously when these compounds were being run on a routine basis. Flow split factor was 1/2. Duplicate samples were taken from chamber #1, chamber #2 (1/2 conc. #1) and chamber #4 (1/8 conc. #1). One sample was inadvertently lost in transfer.

Results and Discussion

The results of the inhalation chamber measurements are summarized in Table 10. These is a large discrepancy between the concentrations as measured by mass spectrometry and the nominal values previously determined by collecting pesticide samples on nucleopore filters and analyzing by gas chromatography. The measured values are consistently low by about a factor of 6. However, the values are internally consistent. The factor of 6 obtains for both materials, and the flow split factors of 1/2 and 1/8 are in good agreement with the measured values.

One possible explanation for the discrepancy could be that a major portion of the air sampled in the bulb was insufficiently equilibrated with the air flowing in the chambers. Each sampling port was connected to a 20 cm long tube mounted in the conical upper portion of each chamber. Since the air does not flow through this tube, the concentration of the aerosol may be low. However, if the values are recalculated assuming that the sampling tube and stopcock attachment "dead volume" contained zero aerosol, it would only raise the measured values by a factor of ~ 1.6. The presence of any impurities in the mass region of interest would contribute to the counts in the minor peak (unlabeled) and give falsely high values.

Since the nominal values of the aerosol concentrations are based on measurements made many months earlier, it is quite possible that the PTN and MePTN used in the chambers had degraded considerably over time (they were stored at room temperature). In order to elucidate the origin of the

TABLE 10

INHALATION CHAMBER MEASUREMENTS

PARATHION

Nominal conc.*		(x Chamb. #1 conc.)	Measured conc.+	(x Chamb. #1 conc.)	Nom/Meas.
Chamb. #1	250 mg/m^3	(1)	36.5 ± 2.5 mg/m^3	(1)	6.8
#2	125	(0.5)	19.7 ± 0.7	(0.54)	6.3
#4	31.25	(0.125)	6.2 ± 0.3	(0.17)	5.0

METHYL PARATHION

Cham. #1	380	(1)	61.5 ± 3.5	(1)	6.2
#2	190	(0.5)	32.0[a]	(0.52)	5.9
#4	47.5	(0.125)	9.5 ± 0.5	(0.14)	5.7

*Based on earlier determinations by glc; values ± 20%

+Average of 2 samplings

[a]One sample lost

discrepancy, it would be necessary to repeat the experiment with fresh PTN and MePTN, and with an improved sampling geometry, making GC determinations of aerosol concentration at the same time that the mass spectrometric samples are taken.

V CONCLUSIONS

In the first year of this research program we have achieved the following goals:

1) Synthesized multilabeled isotopic variants of parathion and trifluralin, as well as methyl parathion;

2) Developed rapid and efficient methods for the isolation and cleanup of pesticide and herbicide residues in animal tissues, based on high-pressure gel permeation chromatography;

3) Worked out final purification methods for parathion and trifluralin, using either TLC or reversed-phase HPLC.

4) Carried out analyses for these compounds on approximately 200 samples of rat tissues, plasma, urine or feces, most of which were done in duplicate;

5) Made preliminary measurements of the concentration in air of parathion and methyl parathion in the inhalation exposure chambers of the SRI Toxicology department.

It can thus be concluded that the isotope dilution analysis methodology works when applied to the kinds of problems posed by this program. There are, however, a few points that require comment and that will be considered as our program progresses.

The isotopic purity of the compounds was not uniformly high. In the case of d_{10}-PTN, no detectable unlabeled material was present in the carrier. In d_7-TFN, however, the d_0/d_7 ratio was unacceptably high, caused apparently by the presence of some unlabeled n-propyl bromide in the deuterated starting material. The deuterated TFN was, therefore, not used as an isotopic dilutant and was instead administered to the animals, while unlabeled TFN served as the carrier. The kinetics of TFN and d_7-TFN metabolism are probably not very different, for reasons already discussed. In other cases, however, it may be necessary to rigorously avoid such occurences.

We have not encountered any major problems in the extraction and cleanup procedures. High-pressure, multiple-column GPC proved to be an efficient cleanup method for a variety of tissues, and it is expected that it will find numerous applications in pesticide residue analysis.

A sequential HPLC method utilizing both GPC and reversed-phase LLC was very effective in purifying TFN samples for mass spectrometric analysis. This method was not, however, as successful when applied to PTN samples, and for this reason, TLC was used as the final purification step. It is likely that a variety of chromatographic techniques will be called upon as different pesticides and herbicides are analyzed, and we intend to develop a broad capability in this area.

The limiting factors in the analyses of PTN and TFN were chemical impurities in the samples and noise due to scattered ions in the mass spectrometer, respectively. Both of these can, in principle, be reduced. A decrease in the chemical background of PTN can be achieved either with the use of higher purity solvents or by a more efficient chromatographic technique, such as gas chromatography. The scattered ion background can be reduced by addition of a retarding lens near the detector.

The biological information that was obtained can be summarized as follows:

PTN was detected at low nanogram levels in plasma and brain of rats at 1 and 2 hr post-injection with 100 μg/kg of the insecticide, but was not found in the liver, fat, or red blood cells of most of the animals. Unchanged PTN was not detected in tissues of animals sacrificed at later times. These data suggest that PTN undergoes rapid metabolism or reaction in rats and is essentially not stored as such in lipophilic depots.

In contrast, TFN was found at high concentrations in fat and liver following IP injection (500 μg/kg). Traces of the unchanged compound also appeared in urine during the first 24 hr post-injection. Determinations of the fat and liver concentrations of TFN in rats at times as long as 72 hr post-injection suggest a biological half-life for TFN of 20 to 25 hr.

Daily oral administration of TFN at three dose levels (20 μg/kg, 100 μg/kg, 500 μg/kg) for three weeks led to detectable uptake of TFN into fat at the higher dose levels. Small quantities of TFN were also found in feces during this regimen, but the compound was not detected in urine at any time, or in plasma or liver at sacrifice (21 days).

The oral TFN data suggest that the compound is poorly absorbed from and extensively metabolized within the gut. Once absorbed, however, TFN remains in fat for an apparently lengthy time period, as suggested by the IP data. Further study of the distribution and kinetics of TFN in other tissues would provide valuable information concerning the toxicology of this important herbicide.

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APPENDIX

RAW COUNT DATA

Parathion-IP	C_m	C_M	C_{AB}	Spectrometer
1 hr Liver				
control	89	245K	124	all 30° sector
rat #1	70	253K	152	
	71	197K	144	
#2	128	312K	192	
	96	237K	165	
#3	98	264K	163	
2 hr Liver				
control	114	260K	181	
	92	277K	153	
rat #1	178	350K	173	
	84	229K	122	
#2	128	235K	165	
#3	126	261K	143	
	156	253K	150	
1 hr Fat				
control	29	55K	28	
	38	15K	63	
rat #1	124	257K	139	
	94	214K	87	
#2	284	413K	417	
#3	72	135K	95	
	20	31K	36	
2 hr Fat				
control	137	299K	147	
	103	140K	115	
rat #1	177	277K	205	
	89	21K	143	
#2	2014	208K	286	
	2127	253K	232	
#3	329	312K	207	
	148	24K	255	

Parathion-IP	C_m	C_M	C_{AB}	Spectrometer
1 hr Plasma				
control	621	835K	831	all 30° sector
rat #1	2365	1190K	802	
#2	4055	518K	430	
#3	678	1400K	998	
2 hr Plasma				
control	405	470K	354	
rat #1	230	200K	351	
#2	875	1380K	600	
#3	1048	1360K	1012	
1 hr Brain				
control	186	360K	313	
	87	280K	131	
	92	270K	137	
rat #1	170	290K	178	
	194	310K	214	
	125	260K	140	
	157	285K	201	
#2	140	280K	173	
	229	475K	284	
	130	230K	155	
#3	386	461K	345	
	403	390K	197	
2 hr Brain				
control	*	*	*	
rat #1	108	382K	162	
	67	112K	56	
#2	212	258K	141	
	209	312K	100	
	122	154K	69	
#3	66	115K	92	
	63	320K	74	

* Control contaminated by reference spot on TLC plate

Parathion-IP	C_m	C_M	C_{AB}	Spectrometer
1 hr RBC				
control	264	254K	164	all 30° sector
	281	254K	222	
rat #1	323	257K	568	
	277	239K	406	
#2	125	262K	142	
	116	296K	135	
#3	151	262K	142	
	149	260K	151	
2 hr RBC				
control	203	312K	201	
rat #1	145	309K	204	
#2	528	547K	426	
	137	287K	186	
#3	150	316K	207	
	85	181K	127	

Background Correction Factors: Parathion-IP

F_{AV} (Liver): 0.65 ± 0.061

F_{AV} (Fat): 0.869 ± 0.18

F_{AV} (Plasma): 0.945 ± 0.28

F_{AV} (Brain): 0.643 ± 0.043

F_{AV} (RBC): 1.30 ± 0.30

Trifluralin-IP	C_m	C_M	C_{AB}	Spectrometer
4 hr Liver				
control	112	870K	92	all 30° sector
rat #1	313	400K	62	
	219	340K	51	
#2	434	490K	92	
	247	320K	68	
#3	397	417K	71	
	188	180K	29	
8 hr Liver				
rat #1	83	1000K	74	all 90° sector
	153	2440K	153	
#2	560	1400K	129	
	977	2520K	192	
#3	288	1650K	100	
	263	1390K	108	
24 hr Liver				
control	89	484K	75	30° sector
	58	229K	36	30°
rat #1	194	359K	45	30°
	848	2395K	128	90°
#2	237	680K	113	30°
	465	2280K	116	90°
#3	148	450K	77	30°
	375	2236K	104	90°
48 hr Liver				
rat #1	141	2120K	103	all 90° sector
	140	2040K	84	
#2	371	2490K	110	
	239	1740K	88	
#3	365	2360K	108	
	217	1460K	72	
72 hr Liver				
control	33	990K	45	all 90° sector
	24	624K	22	
rat #1	97	1430K	50	
	46	817K	40	
#2	97	1890K	78	
	108	1400K	92	
#3	89	966K	47	
	154	1960K	87	

Trifluralin-IP	C_m	C_M	C_{AB}	Spectrometer
4 hr Fat				
control	63	590K	73	all 30° sector
	71	409K	76	
rat #1	1591	171K	35	
	1021	169K	38	
#2	1396	389K	70	
	633	200K	39	
#3	3349	634K	71	
	1327	277K	51	
8 hr Fat				
rat #1	123	556K	108	all 30° sector
	127	593K	131	
#2	1833	527K	116	
	2805	877K	55	
#3	1543	171K	56	
	1785	226K	78	
24 hr Fat				
control	53	990K	60	all 90° sector
rat #1	15330	2100K	130	
	9322	1290K	85	
#2	36560	1590K	134	
	5380	264K	32	
#3	32450	1088K	147	
	16010	544K	84	
48 hr Fat				
rat #1	12476	1170K	96	all 90° sector
	2922	267K	30	
#2	13662	1740K	102	
	4931	625K	39	
#3	15986	993K	78	
	5755	351K	43	
72 hr Fat				
control	53	977K	42	all 90° sector
	17	395K	20	
rat #1	58352	2400K	220	
	42944	1810K	170	
#2	2302	2140K	130	
	1090	945K	77	
#3	12696	1390K	98	
	6207	683K	52	

Trifluralin-IP	C_m	C_M	C_{AB}	Spectrometer
4 hr Plasma				
control	64	610K	48	90° sector
rat #1	89	440K	42	30°
	26	408K	48	30°
#2	84	587K	68	30°
	54	338K	43	30°
#3	47	450K	57	30°
	24	186K	23	30°
8 hr Plasma				
rat #1	53	394K	53	30° sector
	17	170K	20	30°
#2	45	204K	20	30°
	57	1200K	67	90°
#3	65	490K	60	30°
	111	670K	80	90°
24 hr Plasma				
control	67	930K	39	all 90° sector
	50	730K	63	
rat #1	26	830K	35	
	63	1600K	65	
#2	62	1800K	68	
	82	1600K	60	
#3	101	1700K	100	
	36	570K	32	
48 hr Plasma				
rat #1	68	460K	58	30° sector
	86	570K	74	30°
#2	63	1300K	54	90°
	79	580K	70	30°
#3	49	1040K	41	90°
	66	560K	68	30°
72 hr Plasma				
control	53	1030K	33	all 90° sector
	70	980K	50	
rat #1	48	1150K	44	
	56	1160K	45	
#2	58	1780K	63	
	53	1160K	40	
#3	47	1500K	54	
	78	1600K	75	

Trifluralin-IP	C_m	C_M	C_{AB}	Spectrometer
24 hr Urine				
control	83	490K	80	all 30° sector
	56	400K	56	
rat #1	83	615K	73	
	70	500K	52	
#2	64	420K	50	
	178	780K	115	
#3	83	620K	79	
	57	370K	50	
48 hr Urine				
rat #1	70	2000K	56	all 90° sector
	50	1000K	40	
#2	75	2000K	70	
	53	1400K	63	
#3	31	690K	24	
	39	780K	31	
72 hr Urine				
control	53	1100K	43	all 90° sector
	42	830K	46	
rat #1	16	511K	17	
	47	980K	35	
#2	98	1800K	73	
	16	580K	9	
#3	68	2100K	70	
	57	1500K	61	

Trifluralin-IP	C_m	C_M	C_{AB}	Spectrometer
24 hr Feces				
control	48	250K	46	30° sector
	86	470K	70	30°
rat #1	57	290K	36	30°
	93	600K	90	30°
#2	94	1300K	93	90°
	103	560K	112	30°
#3	92	550K	85	30°
	54	1150K	71	90°
48 hr Feces				
rat #1	68	1540K	56	all 90° sector
	36	1160K	47	
#2	48	1330K	47	
	39	1000K	42	
#3	74	1490K	59	
	38	1790K	54	
72 hr Feces				
control	146	710K	171	30° sector
	68	520K	67	30°
rat #1	23	180K	31	30°
	115	690K	84	30°
#2	65	886K	57	90°
#3	89	730K	74	90°
	17	57K	10	30°

Background Correction Factors: Trifluralin-IP

F_{AV} (Liver): 1.17 ± 0.31

F_{AV} (Fat): 0.97 ± 0.17

F_{AV} (Plasma): 1.49 ± 0.17

F_{AV} (Urine): 1.04 ± 0.13

F_{AV} (Feces): 1.04 ± 0.13

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
CONTROL GROUP				
Liver				
rat #1	19	162K	25	30° sector
#2	26	135K	13	30°
#3	13	83K	15	30°
Fat				
rat #1	67	930K	61	90°
	58	1000K	75	90°
#2	45	720K	29	90°
#3	34	670K	37	90°
	22	150K	46	90°
Plasma				
rat #1	14	135K	18	30°
#2	48	390K	54	90°
#3	36	195K	26	30°
7 day Urine				
rat #1	46	278K	43	30°
#2	36	820K	38	90°
#3	16	91K	14	30°
14 day Urine				
rat #1	35	246K	29	30°
#2	19	110K	12	30°
#3*	684	780K	568	90°
21 day Urine				
rat #1	26	193K	27	30°
#2	14	51K	12	30°
#3	17	127K	26	30°

* high background contamination

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
CONTROL GROUP				
7 day Feces				
rat #1	50	322K	49	30° sector
#2	66	360K	62	30°
#3	33	162K	24	30°
14 day Feces				
rat #1	19	227K	27	30°
#2	35	213K	25	30°
#3	27	130K	20	30°
21 day Feces				
rat #1	22	295K	36	90°
#2	60	410K	51	90°
#3	28	48K	36	90°
	26	143K	24	90°

Background Correction Factors: Trifluralin-Oral

F_{AV} (Liver): 1.22 ± 0.67

F_{AV} (Fat): 0.97 ± 0.41

F_{AV} (Plasma): 1.03 ± 0.32

F_{AV} (Urine): 1.09 ± 0.27

F_{AV} (Feces): 1.06 ± 0.28

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
20 µg/kg GROUP				
Liver				
rat #1	19	490K	22	90° sector
#2	30	476K	28	90°
#3	27	600K	28	90°
Fat				
rat #1	27	530K	17	90°
#2	46	850K	49	90°
#3	28	210K	17	90°
Plasma				
rat #1	22	590K	31	90°
#2	28	450K	24	90°
#3	56	610K	36	90°
7 day Urine				
rat #1	31	126K	21	30°
#2	28	180K	38	30°
#3	19	189K	27	30°
14 day Urine				
rat #1	49	1100K	60	90°
#2	63	1270K	59	90°
#3	63	910K	53	90°
21 day Urine				
rat #1	14	250K	19	90°
#2	9	119K	9	90°
#3	22	367K	27	90°

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
20 µg/kg GROUP				
7 day Feces				
rat #1	11	56K	10	30° sector
#2	32	260K	33	30°
#3	28	194K	31	30°
14 day Feces				
rat #1	38	480K	53	90°
#2	21	372K	19	90°
#3	12	290K	19	90°
21 day Feces				
rat #1	43	480K	48	90°
#2	30	510K	30	90°
#3	7	136K	8	90°
100 µg/kg GROUP				
Liver				
rat #1	22	720K	31	all 90° sector
#2	19	660K	31	
#3	86	1500K	72	
Fat				
rat #1	138	990K	57	
	89	776K	37	
#2	96	810K	66	
	36	470K	28	
#3	325	600K	148	
	149	710K	57	
Plasma				
rat #1	2	160K	9	
#2	13	490K	26	
#3	19	390K	21	

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
100 µg/kg GROUP				
7 day Urine				
rat #1	51	790K	47	all 90° sector
#2	33	635K	36	
#3	52	920K	57	
14 day Urine				
rat #1	52	1030K	44	
#2	34	900K	47	
#3	41	1100K	35	
21 day Urine				
rat #1	43	850K	37	
#2	14	560K	26	
#3	12	150K	10	
7 day Feces				
rat #1	58	710K	58	
#2	24	250K	25	
#3	52	660K	45	
14 day Feces				
rat #1	22	200K	29	
#2	41	720K	50	
#3	32	560K	36	
21 day Feces				
rat #1	43	270K	26	
#2	12	245K	16	
#3	23	330K	21	

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
500 µg/kg GROUP				
Liver				
rat #1	17	54K	16	all 90° sector
#2	51	790K	47	
#3	30	380K	22	
Fat				
rat #1	36	100K	17	
	233	940K	64	
#2	140	1290K	59	
	51	447K	44	
#3	68	403K	29	
	139	856K	42	
Plasma				
rat #1	51	1030K	46	
#2	55	710K	44	
#3	33	720K	42	
7 day Urine				
rat #1	149	1600K	161	
#2	182	680K	104	
#3	72	750K	62	
14 day Urine				
rat #1	76	930K	51	
#2	53	790K	47	
#3	90	1300K	66	
21 day Urine				
rat #1	63	596K	44	
#2	61	620K	46	
#3	39	740K	40	

Trifluralin-Oral	C_m	C_M	C_{AB}	Spectrometer
500 µg/kg GROUP				
7 day Feces				
rat #1	48	275K	26	all 90° sector
	108	700K	53	
#2	53	550K	41	
	37	190K	28	
#3	194	120K	79	
	524	520K	72	
14 day Feces				
rat #1	887	360K	86	
	1068	430K	78	
#2	87	540K	39	
#3	46	630K	45	
	123	710K	75	
21 day Feces				
rat #1	160	260K	83	
	215	430K	34	
#2	172	216K	50	
	423	420K	47	
#3	27	215K	17	